

Open Research Online

The Open University's repository of research publications and other research outputs

HIV-1 Infection in Kenyan Infants: Natural History and T cell Responses

Thesis

How to cite:

Slyker, Jennifer Ann (2007). HIV-1 Infection in Kenyan Infants: Natural History and T cell Responses. PhD thesis The Open University.

For guidance on citations see [FAQs](#).

© 2007 The Author



<https://creativecommons.org/licenses/by-nc-nd/4.0/>

Version: Version of Record

Link(s) to article on publisher's website:
<http://dx.doi.org/doi:10.21954/ou.ro.0000ea43>

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

oro.open.ac.uk

HIV-1 Infection in Kenyan Infants: Natural History and T cell Responses

Jennifer Ann Slyker

Submitted for the degree of:

Doctor of Philosophy, Immunology

Open University

31 October 2007

AUTHOR NO W4455531

DATE OF SUBMISSION 05 NOVEMBER 2007

DATE OF AWARD 20 DECEMBER 2007

Abstract

This thesis describes human immunodeficiency virus type 1 (HIV-1) infection in Kenyan infants. Paediatric HIV-1 infection causes rapid disease progression in the absence of antiretroviral (ARV) therapy. African cohorts have reported the highest rates of mortality, from 20-50% at 2 years of life. Understanding the pathogenesis in HIV-1 infected children is important to the design of prevention, treatment, and vaccination strategies. A cohort of 476 Kenyan children born to HIV-1 infected women was studied longitudinally from the time of birth to 24 months. Despite the provision of ARVs to prevent mother-to-child transmission, 19.4% of infants became infected with HIV-1. Infant HIV-1 infection resulted in persistently high levels of viraemia and rapid CD4 depletion. Cumulative mortality at 2 years was 54%. Peak and set-point HIV-1 viral load, and CD4% at 6 months were predictors of 2-year mortality. Co-infection with cytomegalovirus (CMV) before the age of 1 month was also associated with increased risk of death. Infants with HIV-1 infection had poorly contained CMV viraemia in comparison with HIV-1 exposed uninfected controls. Multicolour flow cytometry was used to describe the phenotype of T cells during primary viral infection. Both CMV and HIV-1 infection resulted in dynamic redistribution of T cell populations. High frequencies of activated, apoptotic vulnerable, differentiating CD8 T cells were observed concurrent to acute infection with either HIV-1 or CMV. Co-infection with both viruses resulted in even more profound changes in cellular phenotype. CD4 T cell phenotype was also affected by acute viral infection, but at a much lower magnitude than observed in the CD8 subset. HIV-1 specific CD8 T cells were studied in a subset of infants using IFN- γ ELISpot assays and tetramer staining. Very high frequencies of HIV-1 specific CD8 T cells were identified with tetramer staining, and these cells resembled adult T cell responses in magnitude and phenotype. ELISpot assays revealed weak responses in infants less than 6 months old that increased with age. These data suggest that HIV-1 specific CD8 T cell responses can be generated during acute infection in infants, but IFN- γ production is lower compared to adult cells. Reduced functional capacity may explain the inability of infant T cell responses to contain HIV-1 viral load.

Acknowledgements

These studies could not have been possible without the hard work of many talented scientists, physicians, and staff members. First of all thanks to my mentors Drs. Tao Dong, Sarah Rowland-Jones and Grace John-Stewart, amazing women I am honoured to have known. You have been amazing teachers, wonderful support, and great friends over the past 10 years. Thank you to Drs Mbori Ngacha and Nduati, who work tirelessly to improve mother-child health in Kenya. Thank you to the CTL study participants. Thank you to Sr. Isavwa and the study paediatricians who somehow managed to extract sufficient blood from the infants to enable all these studies. Thank you as well to Dr. Carey Farquhar for support and friendship settling into Kenyan life 7 years ago! Many thanks to Dr. Barb Lohman, who always does good science despite the best efforts of the Kenya Power and Lighting Company to destroy her work. This team is so lucky to have you. Thanks as well to the rest of the lab team Jenn Mabuka, Chris Crudder, Sammy Wambulwa, Steve Moore, and Anne Maingi. Thank you to as well to Prof. Job Bwayo, Gregory Maitha, Rosemary, Marylyn and the others who were taken from us too soon. Thanks to Dr. Julie Overbaugh for mentorship and support in these studies. Thank you to Sandy Emery and Dana DeVange Panteleeff (also of the FHCRC) for doing the GenProbe viral load and filter paper diagnostic assays. Vincent Emery shared the CMV viral load protocols and provided me with primers, standards and other reagents. Ann Atzberger of the Flow Cytometry Core facility at the WIMM spent hours teaching me how to use the Cyan, fixing the instrument and helping me learn about flow cytometry. Many thanks as well to Lizzie Amyes for additional Cyan coaching. Steve Taylor of the CBRG designed the FACS LIMS database and wrote a PERL script to reformat the FlowJo data. Thanks as well to the IT team at the WIMM for crisis support throughout the years. Thank you to the SRJ lab who helped me constantly and every step of the way. Special thanks to Kerstin Luhn for her great PCR advice, and Yanchaun Peng for help with tetramer refolding. Thanks to Marie Reilly for statistical help, mentorship and STATA support. Thank you to Barb Richardson and Ken Tapia for statistical advice with the survival analyses. Thanks to HD (you know who you are!) for your cache of cells, they've been a great asset to these studies. Thank you to Lisa Schimanski for donating cells, and her efforts to print out this massive thesis! Thank you to the Townsend lab for providing respite from the ASH during those early days and for introducing me to the joy of single malt whisky. Thank you to Andy and Ed for teaching me how to make a proper cup of Tea. Thank you to Melanie Goble for reminding me to pay my tuition and keep my OU registration in order. Thank you to my examiners for evaluating this work. Finally thanks to my family and friends for their love and support over these past 4 years. Good luck to all you who are writing up! This thesis is dedicated to Bwana.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION.....	13
SECTION I. THE IMMUNE SYSTEM	13
The lymphoid organs	13
Cytokines and chemokines	14
Toll-like receptors	15
Innate immunity	16
Adaptive immunity.....	21
SECTION II. THE HUMAN IMMUNODEFICIENCY VIRUS.....	44
Viral classification	44
Organisation of the HIV-1 genome.....	44
Regulation of HIV-1 genome expression	50
The HIV-1 life cycle	51
The early history and epidemiology of HIV-1	52
Origins and diversity of HIV	54
Transmission of HIV-1	56
The natural history of HIV-1 infection	60
HIV-1 infection in adults	60
Paediatric HIV-1 infection.....	65
Immune responses during paediatric HIV-1 infection.....	68
Exposed uninfected individuals.....	72
Development of an HIV-1 vaccine	73
SECTION III. CYTOMEGALOVIRUS AND HIV-1	75
Classification and epidemiology	75
Chronic infection and immunosuppression	76
CMV in HIV-1 infected infants.....	76
STUDY QUESTIONS AND SPECIFIC AIMS:.....	78
CHAPTER 2 THE NAIROBI MOTHER-INFANT COHORT: THE NATURAL HISTORY OF INFANT HIV-1 INFECTION	82
Introduction	82
Materials & Methods.....	83
Results	86
Enrolment and follow-up.....	86
Rates and timing of HIV-1 transmission	86
Viral loads during the first two years of life.....	87
CD4 and immunosuppression.....	89
Infant mortality	90
Discussion	92
CHAPTER 3 PRIMARY CMV INFECTION: A CO-FACTOR FOR HIV-1 DISEASE PROGRESSION IN AFRICAN INFANTS?	97
Introduction	97
Materials & Methods.....	98

Results 100

 Prevalence and transmission of CMV in the Nairobi mother-child cohort 100

 Impaired containment of CMV in HIV-1 co-infected infants 103

 Primary co-infection with HIV-1 and CMV 103

 Infant mortality during co-infection 104

Discussion 107

CHAPTER 4 LYMPHOCYTE DISTRIBUTIONS IN HEALTHY AND HIV-1 EXPOSED KENYAN INFANTS 113

Introduction 113

Materials & Methods 114

Results 122

 Lymphocyte distribution in healthy Kenyan infants from the MIP cohort 122

 Lymphocyte distribution in HIV-1 negative children born to HIV-1 infected women 126

 CMV initiates changes in lymphocyte distributions from HIVexp- children 132

Discussion 135

CHAPTER 5 LYMPHOCYTE REDISTRIBUTIONS DURING HIV-1 INFECTION IN KENYAN INFANTS 141

Introduction 141

Materials and methods 142

Results 143

 HIV-1 infected children in the study 143

 Phenotype frequencies in HIV-1 infected children 143

 HIV-1 induced changes in lymphocyte distributions of infants co-infected with CMV 150

 Lymphocyte activation and co-infection with CMV 156

Discussion 158

CHAPTER 6 HIV-SPECIFIC IMMUNE RESPONSES DURING PRIMARY HIV-1 INFECTION 164

Introduction 164

Methods 166

Results 172

 Frequencies of tetramer-stained cells during HIV-1 infection in infants 172

 Phenotype of tetramer positive cells 176

Discussion 179

CHAPTER 7 NEW METHODS 183

Section 1. Synthesis of the novel HLA-A*2902 tetramer 184

 Introduction 184

 Methods and results 186

 Generation of the recombinant HLA-A*2902 heavy chain clone 186

Mini-expression to compare protein expression in clones 191

Protein expression and purification..... 193

Refolding of HLA-A*2902 heavy chain with β_2 microglobulin and gp120 peptide..... 194

Purification of protein monomer 195

Confirmation of biotinylation 197

Generation of tetramers 199

Optimisation of A29 tetramer staining 200

Fluorochrome labels..... 203

Section 2: Optimisation and validations for multicolour flow cytometry 206

General Introduction 206

Are data collected on two different instruments comparable? 207

Methods 207

Results 208

Discussion 215

Titration of antibodies..... 217

Controlling and monitoring of experimental error 219

Reduction and monitoring of inter-experiment and inter-patient variability 220

Quality control..... 221

Isotype controls 224

High throughput methods 227

Gating strategy 227

Data management system 230

Processing of FCS files..... 230

FACS LIMS Database 234

General conclusions 240

CHAPTER 8: DISCUSSION242

REFERENCES247

List of Tables

Chapter 2.

- 2.1 Summary of HIV-1 viral load indicators in HIV-1 infected infants.
- 2.2 Immunologic indicators of immunosuppression in the cohort.
- 2.3 Indicators of HIV-1 disease progression in infants dying before or surviving two years of life.

Chapter 3.

- 3.1 Point-prevalence of CMV viraemia in the Nairobi mother-child cohort during two years of follow-up.
- 3.2 Mean survival times of children stratified by time of CMV viraemia and HIV-1 set-point viral load.
- 3.3 Children with low HIV-1 set-point and late CMV acquisition have the best prognosis during the first two years of life.

Chapter 4.

- 4.1 Antibodies used in immunophenotyping studies.
- 4.2 Antibody panels used to describe cell phenotype.
- 4.3 Lymphocyte subsets examined in the study.
- 4.4 Summary of follow-up and CMV detection in HIV-1 infected children.
- 4.5 CD4 lymphocyte distributions in health children born to HIV-negative women.
- 4.6 CD8 lymphocyte distributions in health children born to HIV-negative women.
- 4.7 CD4 phenotypes in HIVexp- infants.
- 4.8 CD8 phenotypes in HIVexp- infants.

Chapter 5.

- 5.1 Summary of HIV-1 infected infants patient characteristics.
- 5.2 CD4 phenotypes in HIV+ infants.
- 5.3 CD8 phenotypes in HIV+ infants.

Chapter 6.

- 6.1 Peptides used in IFN- γ ELISpot assays.
- 6.2 Antibody mixes used in the description of tetramer-stained cells.
- 6.3 Antibody clones and suppliers.
- 6.4 ELISpot and tetramer staining in HIV-1 infected infants of the CTL cohort.
- 6.5 Summary of HIV-1 specific CD8 cell phenotypes during HIV-1 infection.

Chapter 7.

- 7.1 Pair-wise comparison of ADP and LX estimates of subset frequencies.
- 7.2 Pair-wise comparison of ADP and LX estimates of MFIs.

List of Figures

Chapter 2.

- 2.1 Timing of HIV-1 infection in the CTL cohort.
- 2.2 Plasma HIV-1 RNA viral loads during the first two years of life.
- 2.3 CD4 values and viral loads in children surviving or dying during the first two years of life.
- 2.4 The natural history of HIV-1 infection in adults and infants.

Chapter 3.

- 3.1 CMV viral loads during primary infection in HIV-1 infected and exposed uninfected infants.
- 3.2 Peak CMV viral loads in children born to HIV-1 infected women.
- 3.3 Longitudinal CMV viral loads in co-infected children during the first two years of life.
- 3.4 Correlation between CMV and HIV-1 viral load.
- 3.5 Predictors of mortality in co-infected infants.

Chapter 4.

- 4.1 Gating to select CD4 and CD8 T cell subsets.
- 4.2 CMV infection initiates changes in co-stimulatory phenotype.
- 4.3 CMV infection results in decreased CCR7 and increased CD57 frequencies.
- 4.4 CMV infection results in activation of CD4 and CD8 T cells.
- 4.5 The frequency of CD38^{bright}HLA-DR+ CD8 T cells correlates with CMV viral load during acute infection.
- 4.6 Apoptotic vulnerability during CMV infection.
- 4.7 Lymphocyte redistributions during CMV infection.

Chapter 5.

- 5.1 CD27/CD28 phenotype in the CD4 and CD8 subsets during acute infection.
- 5.2 Early, intermediate and late phenotype frequencies for HIVexp-CMV+ and HIV+CMV+ infants
- 5.3 HIV-1 and CMV initiate differential changes in CCR7 and CD57 T cell phenotype
- 5.4 CCR7+, CD57+, and CD45RA+ frequencies phenotype frequencies in HIVexp-CMV+ and HIV+CMV+ infants
- 5.5 Distribution of phenotypic subsets within CD45RA+/- subpopulations in HIV-1 infected infants.
- 5.6 CD71 expression in HIVexp-CMV+ and HIV+CMV+ infants.
- 5.7 Lymphocyte activation during HIV-1 and CMV infection.
- 5.8 Lymphocyte activation in HIVexp-CMV+ and HIV+CMV+ infants.
- 5.9 Vulnerability to apoptosis during HIV-1 and CMV infection.
- 5.10 Vulnerability to apoptosis in HIVexp-CMV+ and HIV+CMV+ infants.
- 5.11 Activated cells are correlated with HIV-1 viral load during acute and chronic infection.
- 5.12 CMV co-infection and T cell activation at 1 month of age.

5.13 Lymphocyte redistributions during HIV-1 and CMV co-infection.

Chapter 6.

- 6.1 Tetramer staining of CTL-B1-093 from 3 to 24 months.
- 6.2 Tetramer staining cell frequencies decrease over time.
- 6.3 HIV-1 specific immune responses and HIV-1 viral load in HIV-1 infected infants.
- 6.4 HIV-1 specific CD8 cells have a phenotype distinct from the greater CD8 subset.
- 6.5 Summary of co-stimulatory molecule expression in infants with HIV-1 infection.
- 6.6 Expression of activation markers on HIV-1-specific CD8 T cells.
- 6.7 CD45RA+ and CD57+ cells in HIV-1-specific CD8 T cells.
- 6.8 Perforin content is low in HIV-1 specific CD8 T cells.

Chapter 7.

- 7.1 Generation of recombinant HLA-A*2902 heavy chain.
- 7.2 Alignment of clone 3 with HLA-A*290201 reference sequence.
- 7.3 SDS-PAGE to compare protein expression of A*2902-PET clones.
- 7.4 FPLC of refolded monomer.
- 7.5 ECL to confirm biotinylation of tetramers.
- 7.6 HLA-A*2902 tetramer staining.
- 7.7 Different fluorochromes for tetramer and CD8 staining.
- 7.8 Comparison of cell frequencies obtained from ADP and LX flow cytometers.
- 7.9 Cells stained with the same antibodies have different MFI measurements on the ADP and LX flow cytometers.
- 7.10 Linear models for MFIs estimated on the LX and ADP instruments.
- 7.11 Titration for CCR7-PE antibody.
- 7.12 Illustration of PE-Cy7 degradation.
- 7.13 Example of isotype controls to aid in the setting of positive/negative cut-offs.
- 7.14 Placement of gates on 2-dimensional dot plots.
- 7.15 Reformatting of flow cytometry data.
- 7.16 Tables in the FACS LIMS database.
- 7.17 User interface for the Panel Query.
- 7.18 The Panel Query can be easily modified by the user to extract additional data from the database tables.
- 7.19 Schematic diagram of the data management system used in this study.

List of Abbreviations

AC7	APC-Cy7
APC	allophycocyanin
ARV	antiretroviral
AZT	azidothymidine
AIDS	acquired immunodeficiency syndrome
CD	cluster of differentiation
CDC	Centers for Disease Control and Prevention (USA)
CMV	cytomegalovirus
CTL	cytotoxic-T-lymphocyte
Cy	cyanin molecule
DC	dendritic cell
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
FCS	foetal calf serum
FHCRC	Fred Hutchinson Cancer Research Center
FITC	fluorescein
FPLC	fast liquid protein chromatography
FSC	forward scatter
GoK	Government of Kenya
HD	healthy donor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HAART	highly active antiretroviral therapy
IFN	interferon
Ig	immunoglobulin
IL	interleukin
KNH	Kenyatta National Hospital
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
NK	natural killer cell
MTCT	mother-to-child transmission
NGO	non-governmental organisation
PCR	polymerase chain reaction
PB	Pacific Blue
PBMC	peripheral blood mononuclear cells
PC5 or PC7	PE-Cy5 or PE-Cy7
PE	phycoerythrin
PMT	photomultiplier tubes
OI	opportunistic infection
PCP	<i>Pneumocystis jiroveci</i> pneumonia
RNA	ribonucleic acid
SSC	side scatter
TB	tuberculosis
TCR	T cell receptor
Th	T helper cell
TLR	Toll-like receptor
TNF	tumour necrosis factor

TMP-SMX trimethoprim sulphamethoxazole
WHO World Health Organization

CHAPTER 1: INTRODUCTION

SECTION I. THE IMMUNE SYSTEM

The major functions of the immune system are the discrimination of self from non-self and elimination of foreign pathogens. This section reviews basic concepts in immunology pertinent to the study of HIV-1 infection in infants.

The lymphoid organs

The primary lymphoid organs are the locations of lymphocyte development and selection. B cells are produced in the foetal liver and then migrate to the bone marrow where they continue to differentiate and undergo selection. T cells are generated in the bone marrow and mature and undergo selection in the thymus. With age the thymus shrinks, the cortex may disappear completely but the medullary areas persist, enabling low levels of T cells to be generated during adult life (147, 148).

The secondary lymphoid organs and tissues function to concentrate antigen and maximise APC and T cell interaction. The spleen maintains surveillance and defence against blood borne antigens. Lymph nodes (LN) are sites for cells to survey antigens found in the lymph that have entered through the skin or mucosal surface. The LN contains B cell areas (cortex) and the T cell areas (paracortex). The central medulla consists of T, B, macrophages, and plasma cells. Antigen presenting cells carry antigen into the LN, where they interact with T cells. The lymph nodes are located at junctions of lymphatic vessels to form a network that eventually drains into thoracic duct and into subclavian vein and back into the blood circulation. High endothelial

vessels (HEV) allow traffic of lymphocytes out of circulation and into the LN. Integrins on the endothelial cells (VCAM-1, MadCAM-1 etc) mediate adherence to the endothelium and facilitate lymphocyte trafficking into lymphoid tissue.

Cytokines and chemokines

Cytokines work in an interconnected network to co-ordinate all aspects of the cellular immune response, and provide a bridge between the innate and adaptive arms of the immune system. They are signalling molecules that act in an autocrine or paracrine fashion. Cytokines include the interleukins (IL), interferons (IFN), tumour necrosis factor (TNF), growth factors (GF), colony stimulating factors (CSF) and the chemokines (82). Cytokines may activate cells by engaging their receptors thereby initiating signal pathways involving Src and JAK kinases. IL-1(α and β), IL-2, TNF- α and TNF- β help activate T cells. Conversely, IL-10 and TGF- β decrease cytokine synthesis. IL-2, IL-7, and IL-15 stimulate proliferation of T cells, IL-7, IL-14, and IL-15 stimulate proliferation of B cells. Other cytokines have anti-proliferative function: IFN- α , IFN- β , and IFN- γ (Th1 cells). IL-7 and IL-15 support the maintenance of memory cell proliferation and survival (IL-7 and IL-15) (38) (480). IL-4 and IL-5 produced by CD4 T cells determine antibody isotype switching in B cells. The CSFs induce differentiation, proliferation and migration of granulocytes, macrophages and their precursors. IFN- γ , α , and β increase class I MHC expression to augment antigen presentation during viral infection. IFN- γ binding to T cells results in activation of the JAK-STAT pathway, and alters transcription of genes to support anti-viral defences, including the development and proliferation of Th cells and their effector functions (593).

Chemokines are a subset of the cytokines that mediate cell migration. Chemokines have two receptor binding sites, one to bind the epithelium, one to bind the ligand on the tethered lymphocytes. In this way, cells expressing the correct chemokine receptor are recruited to areas of inflammation. Cytokine receptors are classified based on spacing of cysteine residues, α -chemokines (CXC), β -chemokines (CC), XCR1, and CXCR3R1. CCR5 and CXCR4 receptors are co-receptors for the human immunodeficiency virus (HIV). Chemokines can thus compete for receptor binding with viruses and mutations in chemokine receptor genes can inhibit viral entry (106).

Toll-like receptors

Toll-like receptors (TLRs) also provide an important link between the adaptive and innate immune systems. Phagocytes have pattern recognition receptors (PRR) that bind to pathogen associated molecular patterns (PAMPs) - conserved structures common to many microbes. TLRs are an important part of the system, and are expressed on the cell surface or in the cytoplasm on membrane-bound organelles. Binding of TLRs activates adaptor proteins that initiate signal transduction to recruit NF- κ B(361). The cellular response depends upon the TLR ligated and the adaptor protein, and can result in cellular activation, cytokine production, phagocytosis, or apoptosis. Ten TLRs have been identified in humans. TLR4 was the first identified, it recognises LPS. TLR2 recognises peptidoglycan, TLRs 3, 7, 8, and 9 nucleic acids, and TLR-5 recognises flagellin (529).

Innate immunity

Innate immune responses are generated rapidly and have no immunologic memory. Components of the innate arm of the immune system recognise highly conserved motifs common to extracellular pathogens and their specificity does not change upon secondary challenge.

Complement

Complement was first described in 1901 by the Belgian immunologist Jules Bordet as agents in the serum (“*alexine*”) that had the ability to complement the lytic effects of antibodies against bacteria (59). The complement system is composed of ~30 serum proteins that control inflammatory reactions to extracellular organisms. Their functions include chemotaxis, clearance of immune complexes, cellular activation, and direct antimicrobial defence. The complement system is regulated by the short half-life of the component proteins and the activity of complement regulatory proteins bind C3b and prevent it from triggering downstream reactions. The complement system functions through three pathways working in a signal cascade, so a small number of interactions can initiate a large response. In the classical pathway, C1 protein binds IgG or IgM coupled to antigen(438). The alternative pathway is activated by microbial cell wall components that lack complement regulatory components DAF and MCP. The lectin pathway is activated by the interaction of microbial carbohydrates with the lectins: mannose binding lectin (MBL), ficolin H, or ficolin L (191). The three pathways converge to activate C3 protein. C3 protein binds to bacteria, viruses, and fungi, and initiates phagocytosis by binding to receptors on monocytes, macrophages, and DC. Fragment C3a activates phagocytes and mast cells

and C3b can opsonise microbes. Phagocytes have receptors for complement fragments; these fragments diffuse away from the site of infection and stimulate chemotaxis toward the affected area. C3 activation results in the sequential activation of proteins C3-C9, polymerisation of C9 protein to form the membrane attack complex (MAC), which mediates lysis of target cells by inserting itself into the lipid bi-layers of bacteria or viral envelopes.

Granulocytes

The majority of granulated phagocytes (95%) are polymorphonuclear leucocytes (PML), also known as neutrophils, which recognize protein fragments released when complement is activated (C5a), factors from fibrinolytic and kinin systems, products of other leucocytes and platelets, and bacterial products. Microbes are sequestered within phagosomes that fuse with lysosomes, resulting in destruction of the microbe. Granulocytes can also degranulate if activated through their Fc γ receptors and act as cytotoxic cells. Basophils and mast cells comprise about 0.2% of leucocytes. Degranulation of basophils enhances the inflammatory response against parasites but can also cause allergy. Eosinophils comprise 2-5% of blood leucocytes. Though capable of phagocytosis, their primary function is defence against large pathogens that cannot be phagocytosed (such as helminths and schistosomes) via degranulation. Eosinophils also have a regulatory role in the immune response, the release of histamine and aryl sulphatase inactivates mediators of inflammation.

Monocytes and macrophages

Monocytes have several functions which transverse both the innate and adaptive arms of the immune system. Monocytes and macrophages (monocytes which have migrated into extravascular spaces) express PRRs that which recognise PAMPs. Macrophage PRRs include TLRs, CD36 scavenger (which recognises bacterial antigens and apoptotic cells), CD14 (recognises lipopolysaccharide – LPS), complement receptors, and FcR.

Phagocytosis results in degradation of the microbe, and shuttling of microbial proteins into the antigen processing and presentation machinery of the cell, making them important antigen presenting cells (APC) for lymphocytes. When activated by their receptors, monocytes produce cytokines (IL1-, IL-12, IL-18, TNF- α) that activate other cells of the immune system and chemokines (RANTES, MIP-1 α , MIP-1 β) that attract cells to the site of inflammation.

Dendritic cells

Dendritic cells (DC) are professional antigen-presenting cells. There are two main lineages, the myeloid (mDC) which share a common progenitor with monocytes and expresses CD11c+ and the plasmacytoid/lymphoid (pDC) which differentiates from the common CD34+ progenitor and is CD11c-. mDC are potent stimulators of naïve T cells, and secrete IL-12, pDC produce high levels of IFN- α . Immature DC express low levels of class II MHC and have a high endocytotic capacity. They migrate to the non-lymphoid tissues, and are found in areas exposed to the external environment

such as the skin (Langerhans cells) and mucosa where they encounter antigen. Immature DC take up antigen through macropinocytosis, phagocytosis, or via PRRs such as TLRs and mannose-binding C-type lectin receptors, and migrate to the lymph nodes (LN). En route to the lymph nodes they undergo maturation, begin to secrete IFN- α , and interact with naïve precursor CD4⁺ T cells to initiate the cellular immune response. As DCs mature, they begin to express higher levels of MHC class II and become highly efficient antigen presenting cells. The HIV-1 envelope gp120 binds CD209 (DC-SIGN) on DCs and is transported to LNs to facilitate dissemination and infection of T cells (202).

NK cells

Natural killer (NK) and NK T cells are derived from a common progenitor cell in the thymus. NK cells comprise 15% of blood lymphocytes in adults. NK can be identified by the absence of CD3 and the expression of CD16 and/or CD56. At least two phenotypically and functionally distinct groups of NK cells have been identified, the CD56^{bright}CD16⁻ subset, which secretes high levels of cytokines (IFN- γ , TNF- α) and CD56^{dim}CD16⁺ cells which have high cytotoxic activity and can kill targets without prior sensitisation in a non-MHC restricted manner. NK cells mediate cytotoxicity via two main mechanisms. NK cells recognise targets coated with IgG via CD16 (Fc γ RIII receptor) and are activated to secrete cytokines or degranulate, this process is called antibody dependent cellular cytotoxicity (ADCC).

In the second mechanism, NK cell activity is regulated by a balance of activating and inhibitory signals mediated through surface receptors. Engagement of activating

receptors and activation of immunoreceptor tyrosine-based activating motifs (ITAMs) results in cytokine secretion, degranulation, and induction of apoptosis. However, engagement of inhibitory receptors results in the recruitment of cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) to block activation signals. NK cells express killer immunoglobulin-like receptors (KIRs) containing 2 (KIR2D) or 3 (KIR3D) extracellular Ig-like domains. KIRs with short cytoplasmic tails activate NK cell function and those with long cytoplasmic tails inhibit NK cell function (556). The ligands for KIRs are class I MHC molecules, and recent studies suggests their recognition may also be peptide-specific (533) (228). Other NK activating receptors include CD16 (Fc γ RIII), CD94/NKG2C (ligand is HLA-E), NCR, and ILT-1 (Ig-like transcript 1). The inhibitory receptors include ILT-2 (ligands are HLA-A, -B and G), CD94/NK2GA (ligand is HLA-E). Viruses and tumours down-regulate or modify class I MHC as a mechanism to avoid cytotoxic T cells (CTL) and NK recognise virus-infected and tumour cells by their suspicious lack of class I MHC molecules (“the missing self”). If class I MHC are present, the NK cell receives an inhibitory signal and moves on without killing.

NK T cells

NK T cells are found in low numbers throughout the circulation and tissue. NK T cells express NK lineage markers and have an $\alpha\beta$ T cell receptor (TCR). The NK T cell receptor repertoire is much more limited than the CD4 and CD8 T cell TCRs, and is restricted by CD1d bound to glycolipids. There are two main subsets of NK TCRs, the V α 14 and non- V α 14. V α 14 recognise a marine sponge derived α -galactosylceramide (aGalCer), microbial α -glycuronylceramides and the self antigen

isoglobotrihexosylceramide (iGb3). Epitopes for non-V α 14 TCRs have not been identified. NK T cells super-activate resting DC, causing an upregulation of class I and class II MHC and CD40 expression. NK T cells become activated themselves by the interaction, upregulate CD40L expression, and produce very high levels of both Th1 and Th2 cytokines, including IFN- γ , IL-4 and IL-13.

Adaptive immunity

The adaptive arm of the immune system prevents disease upon re-infection by generating immunological memory. During primary infection with a pathogen, naïve precursor cells are activated by specific antigens and undergo clonal expansion as they acquire effector functions. Upon clearance of the pathogen, a number of the effector cells persist in the host as slowly proliferating, long-lived memory cells. Memory cells respond rapidly upon secondary stimulation with antigen, providing rapid clearance of the pathogen. The adaptive immune system can be divided into humoral immunity, which is mediated by B cells and antibodies, and cell-mediated immunity, which is mediated by T lymphocytes.

Humoral Immunity

B cells are produced in the foetal liver and then migrate to the bone marrow where they continue to differentiate and undergo selection. The humoral system consists of antigen-specific molecules that clear antigen in the blood. B cells comprise between 5-15% of lymphocytes and are defined by expression of the B cell receptor (BCR) and CD19, CD20, and CD22. The BCR is immunoglobulin (Ig), most B cells express IgM and IgD, much fewer express IgA, IgE, IgG. Accessory molecules of the BCR

are CD79a and CD79b, which participate in cell-signalling. Most B cells express MHC II enabling them to act as APC for CD4 T cells. B cell activation and homing are facilitated by ligation of complement receptors for C3b, C3d. B cells proliferate upon activation and acquire effector functions. Plasma cells are terminally differentiated B cells which have lost surface Ig but secrete large amounts of Ig (antibodies). Antibodies are vital to acute antiviral defences, and are able to provide sterilising immunity.

The basic structure of the antibody is two identical light chains and two identical heavy chains, one light chain is attached to each heavy chain and the heavy chains are attached to each other. Each of the antibody chains consists of repeating units of amino acids which fold independently into globular motifs known as immunoglobulin (Ig) domains. Both the light and heavy chains have variable (V_L and V_H) and conserved domains (C_L and C_H). Both light and heavy chains contain hypervariable regions in the N-terminus called complementarity-determining regions (CDRs). CDRs are presented as projecting loops with highly variable amino acid sequences, and the CDR3 regions form the antigen binding site. Proteolytic cleavage of Ig monomers revealed distinct fragments with different binding properties(107). Digestion with papain cleaves Ig before the disulphide bond which links the two heavy chains, resulting in two identical fragments each retaining the ability to bind antigen (Fab = Fragment, antigen-binding). These were formed by the combined light and heavy chains and contained both variable and constant domains of each. The third fragment, formed by the C_H domains, crystallised into a lattice (Fc = fragment, crystalline). The Fc portion of Ig is conserved between antibody classes (isotypes) and mediates many of the effector functions of antibodies. Digestion of Ig with

pepsin cleaved after the disulphide bond, resulting in a large $F(ab')_2$ fragment which contained the two Fab regions. The Fc region was cleaved into small peptides. In summary, each monomeric antibody was determined to have 2 Fab and one Fc region.

Generation of antibody diversity

Stimulation of cells with antigen was shown to result in the expansion of cells producing antibodies against the antigen (550). The germ line was too small to account for this level of diversity on a one gene, one protein basis (535). Comparison of heavy and light chains from different mouse tumour cell lines revealed that half of the chains was always identical within antibody classes, whilst the other was unique, and in 1963 Dreyer and Bennett put forth the hypothesis that the genetic material adjacent to the common part of the antibody was subject to a “genetic scrambling process” during the differentiation of specific cells (152), and that this could be achieved by multiple crossovers during recombination (507). It was later discovered that both heavy and light chains undergo somatic rearrangement. Antibody diversity is achieved by two mechanisms, V(D)J recombination during B cell ontogeny, and somatic hypermutation in response to antigen binding. Multiple genes encode the variable and constant regions of the Ig protein. The light chain variable region is encoded by multiple variable (V) and joining (J) genes and the constant region is encoded by constant (C) genes. The heavy chain variable region is encoded by V, J, C and D (diversifying) gene segments. Somatic rearrangement of the Ig genes begins with joining of D and J genes in the heavy chain locus and deletion of intervening DNA(20). One V gene is joined to the DJ complex to create VDJ. All D segments 5' to the rearranged D are deleted. Following transcription the intron between VDJ and C is spliced out, giving rise to the functional VDJC transcript of the heavy chain. The

light chain follows the same pattern of VJC arrangement, following translation the light chain assembles with the heavy chain and is transported to the cell surface. Recombination of the Ig loci is mediated by recombination activating genes 1 and 2 (RAG-1 and RAG-2) which recognise recombination signal sequences (474) (356) in the introns between the genes. Combination of different heavy and light chains adds another layer of diversity to the Ig repertoire, there are 5 heavy (α , δ , ϵ , γ , μ) and two light chain loci (λ , κ). Finally, junctional insertions and deletions during rearrangement can further add to diversity. Antigen dependent somatic hypermutation of Ig follows ligation of antigen. Point mutations are induced into the V region of the antigen binding site, creating an Ig variant with enhanced affinity for the antigen. B cells expressing Ig for self antigen are deleted in the bone marrow, become anergic, or undergo additional light chain rearrangements to alter antibody specificity (210) (239).

Antibody class is determined by structure of the heavy chain. In secreted form the tails of the heavy chains interact with one another through a 15 kD polypeptide to form multimeric Ig molecules, IgM forms a pentamer and IgA forms a dimer. When bound to the cell membrane all antibodies are monomeric. IgG comprises 70-75% of immunoglobulins and is a monomer in secreted form. IgG is the major Ig involved in secondary immune responses. IgG promotes ADCC by opsonisation, binds Fc receptors on macrophages and neutrophils, and activates the classical pathway of complement. IgM in pentameric, it comprises ~10% of serum Ig and is important in the response to blood-borne infectious organisms, especially during acute infection. IgA forms 15-10% of Ig and exists as a monomer or dimer. IgA protects mucosal surfaces and is abundant in saliva, colostrum, breast milk, and tracheobronchial and

genitourinary secretions. IgD forms <1% of serum Igs, but is a major B cell surface component and is involved in B cell differentiation. IgE is also found in low concentration in the serum (<1%), it is a component of surface membrane of basophils and mast cells. IgE is involved in the sensitisation of mucosal surface cells on conjunctival, nasal, and bronchial mucosae and may prevent helminth infection. IgE is also involved in the generation of allergic reactions.

Affinity maturation and class switching

The processes of affinity maturation and class switching are important in primary and secondary immune challenge (394). In the early stages of the immune response, antibodies have low affinity for antigen. During the course of the immune response the average affinity of antibodies increases in a process called affinity maturation. Two mechanisms mediate this, somatic hypermutation and antigen driven selection and expansion of B clones expressing higher affinity antibodies. The predominance of higher affinity antibodies allows a more rapid, efficient clearance of pathogen upon secondary challenge. Antibody class switching also occurs during B cell proliferation. Though each B cell produces only one type of V_L chain, somatic recombination and differential splicing of the heavy chain constant (C) genes enable variation in the heavy chain. Hence a cell once producing one type of antibody (such as IgM) can switch to producing another class (IgG) of antibody with the same light variable chain and same epitope specificity.

Together, affinity maturation and class switching enable the adaptive immune system to generate a more efficient secondary response to challenge. The primary response to viral challenge occurs after a time lag, characterised by appearance of high IgM

and low IgG titres, with mixed low and high affinity. The secondary response occurs much more quickly than the primary challenge, antibody titres may be >10-fold greater, antibodies are of higher avidity, and primarily IgG-type.

Antibodies in the foetus

Though B cells are present in the developing foetus, antibody production is greatly reduced in the neonate (230). IgM cannot cross the placenta, and is produced by the neonate. IgA production is very low in the newborn, it also does not efficiently cross the placenta and must be acquired through breast milk. Though neonates cannot synthesise IgG, high levels are acquired through placental transfer. By 9 months of age, IgG synthesised by the infant has usually replaced maternal antibody (5), but prolonged breastfeeding can mean the persistence of maternal antibody. By 12 months of age, the infant produces 80% of adult level IgG, 75% of IgM, and 20% IgA.

T lymphocytes

T cells are produced in the bone marrow from and migrate to the thymus where they differentiate and undergo positive and negative selection. T cells are identified by their expression of CD3, CD45 and the surface T cell receptor (TCR). The TCR is a disulphide linked heterodimer of two subunits, either $\alpha\beta$ (95% of T cells) or $\gamma\delta$ chains (~5% of T cells). $\gamma\delta$ T cells protect the mucosal epithelia, their TCRs recognize glycolipids and lipids and do not appear to require APC. Most $\gamma\delta$ in the blood are CD4-CD8-, in tissues they are usually CD8+. $\alpha\beta$ T cells are the main mediators of cellular immunity against viral infection. Each subunit chain contains two Ig-like

domains, α and β , each of which has a variable (V) and constant domain (C) (198). The α and β chains are anchored to the plasma membrane and have a short cytoplasmic tail. Each of the V regions contains 3 complementarity-determining regions (CDR) that bind the peptide and MHC. The TCR is expressed at the cell surface in association with the CD3 molecule. CD3 is necessary for TCR expression at the cell surface and for signal transduction upon TCR ligation. CD4 and CD8 proteins expressed on the T cell form part of the binding complex to recruit tyrosine kinases that phosphorylate CD3, and initiate T cell activation.

T cells are produced in the bone marrow and mature in the foetal thymus. The expression of Notch-1 results in commitment to a T cell lineage (581). Diversity in the TCR repertoire is generated by similar mechanisms as the Ig genes and somatic recombination is mediated by RAG-1 and RAG-2. Genes are rearranged during lymphocyte differentiation in the thymus while the cells are CD4-CD8-. The α chain genes are located on chromosome 14 and is composed of V, J and C segments and the β chain is composed of V, J, C and D segments on chromosome 7. There are two β chain loci and one α chain locus. The β chain is arranged first, D is joined with J then the DJ complex is joined with a V segment to form VDJ. Splicing of mRNA results in joining with a C gene segment (there are two and selection of which one seems to be random). Rearrangement of the α chain locus is similar, V is joined with J, and following transcription, VJ mRNA is joined to C to make VJC. Only one of the two β chain loci are expressed in the T cell, the productive arrangement of one locus inhibits rearrangement of the corresponding locus on the other chromosome by allelic exclusion. TCR diversity is thus achieved through the use of multiple VD and J segments, the combination of different V, D and J genes during rearrangement, and

the pairing of diverse α and β chains. Junctional diversity is also generated through N-region diversification of both the α and β chains which is catalysed by deoxyribonucleotidyl transferase (TdT) (288). This includes the random addition of nucleotides that are not part of the genomic sequence at VD, DJ, and VJ junctions and imprecise joining of gene segments. In addition, many β chain D segments can be translated in all three reading frames. By the combination of these processes, it is estimated that $\sim 10^{11}$ different TCR CDR3 arrangements are possible.

“Double positive” CD4+CD8+ $\alpha\beta$ T lymphocytes undergo selection in the thymus following TCR rearrangement. During positive selection, antigen is presented by DCs and macrophages of the cortical epithelium of the thymus. Lymphocytes with TCR that recognise self-MHC are given a survival signal, those that fail to recognise self MHC undergo apoptotic death (death by neglect). During negative selection TCRs with high affinity for self MHC/peptide complexes are deleted. The majority of self-reactive T cells are deleted during this process, and only a small fraction of the original TCR repertoire survives the process to enter the circulation. Based upon their recognition of class I or class II MHC and the strength of signal received, CD4 or CD8 is lost from the double positive thymocytes, and the mature cells express CD4 or CD8 (252, 404).

T cell subsets

Though they share a common progenitor, CD4 and CD8 T cells have distinct properties. CD8 T cells recognise infected cells by the presentation of viral antigen complexed to class I MHC molecules. Once activated, CD8 T cells proliferate and mature into effector T cells. Mature cytotoxic CD8 T cells (CTL) can kill virus-

infected cells via Fas or perforin-mediated pathways. CD8 T cells also release cytokines which promote their own proliferation (IL-2) and limit viral infectivity (IFN- γ). Cells of this phenotype are called Tc1. Alternately, CD8 T cells may produce suppressive cytokines (Tc2 cells) such as IL-10 which inhibits cellular proliferation and activation by down-regulating cytokine synthesis.

CD4 cells can also be cytotoxic but primarily support the adaptive immune response via the production of cytokines that stimulate CD8 T cells, B cells and DC, hence the name “T-helper” cell. CD4 recognise APC expressing class II MHC. CD4 are further categorised by their production of IFN- γ (Th1) or IL-4 (Th-2). The polarisation of naïve CD4 into Th1 or Th2 cells is determined at the time of priming by the strength of TCR-MHC peptide engagement and cytokine signalling (530, 531) (400). CD4 can be induced into a Th1 phenotype by stimulating naïve cells in the presence of IL-12 (*in vivo* this comes from DCs), and Th-2 type can be induced by stimulation in the presence of IL-4. Polarisation to a Th1 phenotype is mediated by the action of STAT4 and T-bet which induce the IFN- γ gene (527) (383). Alternately STAT6 signalling and GATA3 induce the IL-4 gene resulting in a Th2 phenotype cell (314). Th1 cells are important in defence against intracellular pathogens, their IFN- γ activates macrophages, and binding of CD40L on T cells to CD40 activates DCs. Th1 cells also produce IL-2, which stimulates cellular proliferation. Th2 type cells produce IL-4, IL-5, IL-6, and IL-10. Th2 cells are active against extracellular pathogens, they mediate inflammation and stimulate B cells to proliferate and secrete antibodies. Follicular B helper T cells (T_{FH}) are a distinct subset of CD4 T cells that support the development of memory B cells in lymphoid follicles (557). T_{FH} express CXCR5 and thus home to germinal centres of B cell proliferation. T_{FH} may develop

from Th2 type cells, or may form a separate lineage; commitment to the T_{FH} phenotype is believed to be determined by the expression of transcription factor BCL-6. T_{FH} from the follicles of the tonsils express the inducible T cell co-stimulator (ICOS), OX40 (CD134), CD40L, and secrete IL-10 and IL-21, which together support B cell survival and development. ICOS ligation stimulates T cells to proliferate and produce IL-10, which with IL-21 enhances B cell proliferation. B cells failing to interact with T cells undergo apoptosis, but ligation of CD40 results in survival. OX40 binding is required for the development of memory plasma cells, somatic hypermutation and antibody class switching. The presence of T_{FH} in B cell germinal centres may thereby enhance B cell proliferation, survival, and development into long-lived memory plasma cells. Additional CD4 T cell subpopulations have been defined by their ability to secrete high levels of TGF- β (Th3), and IL-17 (Th17) (232). Th3-type cells have suppressor function, and Th17 are important in inflammatory responses at mucosal surfaces(283).

CD4 T cells also have suppressive functions that are important in the maintenance of peripheral tolerance and down-regulation of the adaptive immune response(463). These regulatory T cells (Tregs) can be divided into naturally occurring (nTreg) and induced (iTreg). Both have the ability to down-regulate CD4 and CD8 activity, including the production of IFN- γ and proliferation. nTreg express high levels of the IL-2 receptor α (CD25), glucocorticoid-induced TNF receptor (GITR), and CD152 (CTLA-4), are CD127^{low} and are dependent upon IL-2. nTreg are committed in the thymus to a regulatory function by induction of the FOXP3 gene (a fork head/winged helix family gene) transcriptional regulator(199, 280). This subset functions in a non-antigen-specific manner, though signalling through the TCR may be involved in their

activation. nTreg are important in maintaining peripheral tolerance to self-reactive T cells that have escaped thymic selection; in the mouse model their absence results in autoimmunity and adoptive transfer has been shown to restore peripheral tolerance(464). The suppressive mechanism of nTreg activity is not yet well defined. *In vitro*, nTreg appear to require cell-cell contact, but suppressive cytokines (IL-10 and TGF- β) may also be involved. iTreg are generated during the adaptive immune response, and arise from naïve CD4 T cells via induction of Foxp3. The generation of iTregs is antigen-specific, and requires the participation of cytokines including TGF- β . The iTreg subset is more heterogeneous than the nTreg, and includes CD4 cells producing IL-10, and TGF- β .

MHC restriction

The importance of cytotoxic T cells in antiviral immunity was revealed during the early 1970s with the demonstration that splenocytes isolated from mice infected with lymphocytic choriomeningitis (LCM) or ectromelia (mouse pox) viruses could reduce viral titres when adoptively transferred into mice or used *in vitro* lytic assays (51-53, 373). Anti- θ serum reduced or ablated the effect suggesting the transferred cells were derived from the T cell lineage. Experiments by Zinkernagel and Doherty revealed that histocompatibility between at least one H-2 allele of the effector and the target cells was required for the lytic activity (601, 602). This led to the “altered self” hypothesis that the transferred cells recognised either virus-modified H-2 or virus complexed with H-2 (601), though it was still a matter of debate as to whether there was one or two receptors (one for the virus, one for the MHC) involved in the signal. In humans HLA restriction was confirmed using influenza virus (360). The final pieces of the puzzle were assembled with the crystallisation of the HLA-A2 molecule

(48) (49) and the demonstration that T cells recognise epitopes on viral protein fragments presented by HLA molecules(541) (539, 540).

The human HLA locus is located on chromosome 6 and encodes several genes involved in the immune response. The class I genes encode HLA-A, B, C, E, F, G. The class II genes encode HLA-DRA, DRB, DPA, DPB, DQA, and DQB genes. The class III genes encode several diverse genes, many of which are involved in immunity; these include complement proteins, TNF- α , heat-shock proteins and proteins involved in antigen processing (TAP). HLA-A, B, and C present small viral peptides to the CD8 T cell receptor. HLA-C, E and G are NK cell ligands, the expression of HLA-G on the placenta inhibits NK-mediated rejection of the foetus(451). The class II HLA molecules present viral peptides to the CD4 T cell receptor.

Class I HLA molecules are expressed on nearly all nucleated cells. The class I MHC molecule is a heterodimer composed of a heavy 45kDa chain and the non-covalently bound, light invariant β_2 microglobulin chain(469). There are three extracellular domains, $\alpha 1$ (N terminal), $\alpha 2$, $\alpha 3$ and a cytoplasmic tail. The $\alpha 3$ domain interacts with CD8 on the T cell and is invariable. Together $\alpha 1$ and $\alpha 2$ together form the antigen-binding groove that holds the viral peptide. The peptide-binding cleft is formed by two alpha helices atop a β -pleated sheet, residues in both the helices and the sheet are polymorphic and differ between HLA-types. These variations form the 3-D structure of the groove and determine what peptides may bind there. The floor of the peptide-binding groove has recessed pockets with amino acid motifs enabling the binding to specific amino acid side chains. There are two pockets in the binding

groove that are critical for stable peptide binding, these bind “anchor residues” at position 2 and 9 of the peptide(309, 524). Though each individual HLA molecule may have the potential to bind multiple peptides, the array of peptides that bind there is limited by the anchor residues. Changes to anchor residues in the peptide are most likely to inhibit binding. Peptide side chains point upwards away from the HLA molecule and these are recognised by the TCR. Changes in amino acid sequences that lie at anchor residues, or contact the TCR can impede binding to the HLA or the TCR, and is one of the mechanisms by which HIV-1 escapes from CTL responses. The class I peptide binding groove is closed on its ends so the length of peptides binding there is limited to 9-11 amino acids. Class II MHC are expressed on a limited number of cells including macrophages and DC. Class II MHC are heterodimers of 2 heavy chains, comprised of $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$ extracellular domains; there is no $\beta 2$ microglobulin subunit attached. The overall structure of the peptide binding cleft is similar to the class I molecule, but the antigen binding cleft is open on the end, enabling it to accommodate larger peptides over 20 amino acids long(522).

Antigen processing and presentation to T cells

Antigen presentation via class I and II MHC requires “processing” of foreign proteins to generate peptides of the appropriate size. Though any cell expressing class I or II MHC has the potential to be an antigen presenting cell (APC), some cell types are particularly efficient at processing antigen and presenting it to T cells. These are known as “professional” APC; these include B cells, macrophages, and DC. DCs are the most effective APC, and unlike B cells and macrophages their primary function appears to be antigen presentation.

Antigens for class I MHC are cellular proteins produced endogenously within the APC. Sources of endogenous protein include viral and host proteins synthesised within the cell, alternative transcripts and defective proteins. Peptides have been found with post-translational modifications suggesting that stable proteins can also be presented through the endogenous pathway. Proteins are targeted for degradation by ubiquitination and degraded within the barrel-shaped proteasome by the LMP2 and LMP7 subunits(99, 538). Following egress from the proteasome, peptides are further trimmed at the N-terminus by cytosolic peptidases. TAP proteins 1 and 2 stabilise the peptides to prevent degradation in the cytosol while being transported to the lumen of the ER. In the ER more trimming may take place via ERAAP or ERAP1 peptidases. Chaperone proteins calnexin, calreticulin and ERP57 associate with the class I MHC molecule while awaiting assembly. TAPASIN is involved in quality control for MHC/peptide assembly, it mediates selection of peptides for loading based on their half-life, retains unstable complexes in the ER, and may retrieve unstable complexes that have already exited the ER(243, 416). TAPASIN forms a bridge between TAP and the MHC until the peptide is able to associate with class I. B2microglobulin attaches to the MHC, and peptide is loaded before disengagement of chaperones. More peptide editing may take place after loading on the MHC. Association of all three components, heavy chain, β 2microglobulin and peptide, are required for expression at the cell membrane (537). The assembled peptide-MHC complex is transported through the Golgi to the plasma membrane.

T cell priming of class I MHC to exogenously derived antigen has also been described(46). This so-called “cross-presentation” enables the presentation of antigens which do not infect the APC, and is particularly important in immunity

against tumour antigens. DC and macrophages are both capable of cross presentation. Antigen endocytosed via Fc γ R, phagocytosis or macropinocytosis is exported to the cytosol for proteasomal degradation (294) then escorted by TAP for loading onto class I MHC in the ER or in specialised phagosome-ER compartments (221).

The class II pathway presents proteins that have been endocytosed by phagocytosis, macropinocytosis or receptor-mediated endocytosis, and processed in endosomes. In addition to microbial antigens, apoptotic cells also enter into the class II system in this way. Following translation, the $\alpha\beta$ -heterodimer is inserted into the ER membrane and complexed with an invariant polypeptide (Ii) which serves as a placeholder until peptide loading (115). Class II MHC are assembled in a specialised compartment called the MIIC. The Ii fragment is cleaved into smaller fragments, one of these the CLIP (class II associated invariant peptide) sits in the peptide-binding groove of the class II molecule until it is replaced by peptide. It is not yet known whether proteins are degraded before or after loading onto the MHC (491). The HLA-DM molecule binds to the class II MHC, altering its binding properties and enabling release of CLIP and allowing peptide to take its place (567). The entire complex is then transferred to the cell membrane for presentation.

The immunological synapse and cellular activation

The immunological synapse is formed by contact between an APC and an effector cell. The TCR is angled at 45-80° across the MHC binding site. The CDR3 loops of the V β chains form the peptide binding site and the CDR1 and bind the MHC (41) (139). Only a few amino acids of the peptide are contacted by the TCR at the interface, between 5-7 on class I and 6-7 on class II peptides. Changes to the peptide

amino acid sequence can greatly alter binding at the interface; altered peptides may fail to load onto MHC or contact TCR (no delivery of activation signal), may have reduced avidity for TCR (delivering a weakened signal) or may act as antagonists, inhibiting T cell activation. T cell activation corresponds to the “tightness” and the duration of the binding (118).

The participation of other molecules is also crucial for delivery of an activating signal. During TCR-MHC engagement, the microtubule organising centre (MTOC) becomes polarised to a location underneath the synapse. Other molecules within the T cell membrane are recruited to the area of TCR/MHC interaction by the movement of lipid rafts. A “bulls-eye” pattern is formed with the TCR and associated molecules forming the supramolecular activation complex (cSMAC) at the centre, surrounded by adhesion molecules and CD45 (244). Adhesion molecules on the APC (ICAMs -1 (CD54) ICAM-3 (CD50)) interact with LFA-1 (CD11a/CD18) and talin on the T cell to increase the duration of interaction between the two cells. Complete T cell activation requires delivery of a secondary signal after TCR-MHC interaction, this signal is provided by co-stimulatory molecules. CD28 on T cells interacts with B7-1 & B7-2 (CD80 & CD86) on DCs or monocytes. Alternatively, CTLA-4 can interact with B7 molecules to deliver an inhibitory signal. Co-stimulation via CD28 greatly augments the activation phenotype of the cell, particularly IL-2 production. If no second signal is delivered, T cells do not respond optimally to antigen, and antagonism can result.

The process of cellular activation proceeds from the secondary receptor engagement through intracellular motifs. CD3 is associated with the TCR at the cell membrane

and its tail contains an immunoreceptor tyrosine activating motif (ITAM). When TCR is engaged, the ITAM is phosphorylated by p56 Lck, and results in the recruitment of ZAP-70. Zap-70 phosphorylates downstream proteins to begin a signal transduction cascade. This cascade ultimately results in the release of Ca^{2+} ions from intracellular stores and translocation of transcription factors (NF-AT and NF- κ B) to the nucleus (174). NF-AT activates IL-2 transcription, which has both autocrine and paracrine function to initiate cellular proliferation.

Mechanisms of cytotoxicity

Cytotoxic T cells (CTL), NK, and cytotoxic CD4 can all participate in the cytotoxic response. Cytotoxicity can occur via direct cell-to-cell signalling or via soluble intermediaries. All pathways involve cellular differentiation into effectors (activation, proliferation & formation of cytolytic granules), unidirectional delivery of a death signal, and initiation of apoptosis in the target cell. CTL and NK are serial killers, with cell-cell interactions occurring within a few minutes, whereas killer CD4 maintain a single cytotoxic synapse for several hours. Two main pathways predominate in the adaptive cytotoxic immune response, the Fas and granule-mediated pathways (267). Activated CD4 and CD8 T cells upregulate expression of Fas ligand (FasL), enabling delivery of a death signal to cells expressing Fas receptor (CD95). FasL may be stored in cytolytic granules (and secreted) or expressed at the cell surface. TNF receptor 1 (TNFR-1) pathway is triggered by TNF and lymphotoxin, which are stored in the vesicles of CTL, NK and macrophages. Like Fas-FasL signalling, the death signal is delivered when TNF or lymphotoxin encounter the receptor on the target cell. Though different receptors and ligands are involved, the signalling pathways overlap. Receptors are trimerized after ligation and

an intracellular death-inducing signalling complex (DISC) is formed as death domains within the target cell associate with other proteins that recruit and activate caspases 8 and 10.

Granule-mediated killing occurs through the formation of a cytotoxic synapse between killer and target cell. Upon activation of the killer cell, cytotoxic granule formation increases. Downstream signalling in the effector cell results in the mobilisation of Ca^{2+} and reorganisation of the cytoskeleton. Granules become polarised in the effector cell toward the target cell membrane. The final step in formation of the synapse is fusion of cytotoxic granules with the cell membrane and Ca^{2+} -dependent exocytosis. Several proteins are contained in the cytotoxic granules that enable unidirectional cytotoxicity. Perforin polymerises in Ca^{2+} -dependent manner and forms pores in cell membranes (594). Calreticulin binds perforin and may protect perforin-mediated damage to the effector cell (189). Cathepsin B degrades membrane-bound perforin and may protect the effector cell from membrane damage (34). Granzymes are serine proteases that enter the target cell cytosol to activate apoptosis. Though it was previously believed that granzymes entered the target cell through perforin channels in the cell membrane, this is now thought unlikely because granzymes are much larger than the pore formed by perforin (72). Further experiments suggested that granzyme and perforin are likely to be endocytosed by the target cell and perforin then facilitates the release of granzyme into the target cell cytosol by disrupting the membrane of the endosome. Granzyme A then translocates to the nucleus to directly initiate apoptosis via the SET, a complex of multiple proteins which interact with granzyme A to nick DNA. Granzyme A also disrupts the nucleosome to make DNA more susceptible to degradation by DNases.

Granzyme A can also cleave lamins A-C to disrupt the nuclear lamina. Granzyme B induces apoptosis differently, utilising the apoptotic pathways within the target cells. Its substrates are anti-apoptotic proteins, and their cleavage releases cytochrome c from the mitochondria. Cytochrome c initiates formation of the apoptosome and initiates a cascade of events culminating in activation of caspases and DNA fragmentation.

Changes in T cell phenotype during viral infection

The activation of T cells results in the acquisition of functions that enable the clearance of viral infection. T cell activation and maturation into effector cells can be observed by the differential expression of cellular proteins during the immune response.

Cellular activation

CD69 is the earliest glycoprotein upregulated on the cell surface after T cell or NK activation. CD69 is an activating receptor and its ligation increases Ca^{2+} mobilisation, cytokine and cytokine receptor synthesis. Its expression is transient and its natural ligand has not yet been identified. CD38 is expressed on NK, B and T cells and is upregulated upon cellular activation (18, 439). CD38 is constitutively expressed upon neonatal T cells, and its presence on a T cell is not in itself an indicator of activation(341, 497). CD38 has multiple functions that support cellular activation. The enzymatic activity of CD38 produces products involved in the regulation of cytoplasmic calcium levels. CD38 catalyses the conversion of NAD^+ to nicotinamide (NAm) and cyclic ADP-ribose (cADPR), and the degradation of cADPR to ADP-ribose (ADPR)(363). CD38 may be directly involved in signalling.

Interaction of the cytoplasmic tail with Ick results in the mobilisation of Ca^{2+} and the production of cytokines (32). CD38 also functions as an adhesion molecule between leucocytes and endothelial cells by interacting binding CD31 (124).

CD95

Engagement of the T cell receptor results in spontaneous apoptosis of T cells in the absence of “rescue” stimuli, in a process called activation-induced cell death (AICD) (17, 73, 133, 266). Apoptotic death is important during the contraction phase of immune responses to limit inflammatory responses that could become dangerous to the host. T cells also express CD95 (Fas) on the surface following activation, making them susceptible to apoptosis upon encounter with the Fas ligand (FasL, CD178). Additionally, the down-regulation of anti-apoptotic factor Bcl-2(242) is involved in the Fas apoptotic pathway (251, 592). During the contraction phase of viral infection, high frequencies of cells are detected expressing CD95, with down-regulation of Bcl-2 and these are highly susceptible to apoptosis(64). CD95+ cell frequencies are elevated in patients with HIV-1 infection.

Co-stimulatory molecules

Changes in the expression of co-stimulatory molecules occur following cellular activation. CD28 delivers a second signal during activation through the TCR via interaction with its ligand CD80 (or CD86). CD27 is a TNF receptor family molecule required for T cell effector and memory cell development. The ligand of CD27 is CD70, which is expressed upon T cells, B cells and DCs. In a mouse influenza model, CD27-CD70 signalling promotes the survival of activated T cells, increasing the number of effector cells at sites of infection (238). CD27 is also important in

generating a secondary response to stimulation of memory CD8 T cells (237). During viral infections in humans, CD27 and CD28 have been used together to describe the effector and memory status of virus-specific CD4 and CD8 T cells. CD4 and CD8 cells are proposed to follow a unidirectional pathway of maturation from a naïve to effector and memory phenotype that can be tracked by the expression of CD27 and CD28. Naïve or early effector phenotype cells are CD27⁺CD28⁺, stimulation with antigen results in the sequential loss of CD27 and CD28 to a double negative “late” CD27⁻CD28⁻ phenotype. CD4 lose CD27 expression first, followed by CD28, and CD8 lose CD28 expression first followed by CD27, and these single positive cells have thus been called “intermediate”. Comparison of antigen-specific CD8 T cells during acute infection with HIV-1, Epstein-Barr virus (EBV) and hepatitis C virus (HCV) has revealed that most responding cells are CD27⁺CD28⁺, low in perforin content, high in activation markers, low in Bcl-2 and proliferating (26). In contrast, during chronic infection the virus-specific cells were enriched in different subsets. EBV and HCV-specific CD8 were primarily early (CD27⁺CD28⁺), HIV-1-specific CD8 T cells were primarily intermediate (CD27⁺CD28⁻) and cytomegalovirus (CMV)-specific CD8 were predominantly late (CD27⁻CD28⁻) phenotype cells. Though expression of CD27 and CD28 was different for these viruses, all appeared to be effector T cells capable of cytokine secretion and cytotoxic activity. However, HIV-1 specific CD8 appeared to be less functional than other virus-specific T cells; HIV-1 specific CD27⁺CD28⁻ were able to secrete cytokines, but had low levels of perforin and were less capable than CMV-specific CD8 T cells in lytic assays (27). Selective expansions of CD27/CD28-subpopulations of cells have also been observed in the CD4 subset. In CD4 T cells, EBV-specific CD4 T cells are predominantly

early phenotype, and CMV-specific CD4 T cells are a mixed phenotype of intermediate and late (24).

Proliferation

Cellular proliferation results in the clonal expansion of virus-specific effector T cells. Brief exposure to antigen results in cellular proliferation, the development of full effector function, and the development of memory. However, persistent stimulation results in clonal exhaustion, loss of effector function, and apoptosis. The impairment of CD4 proliferation during persistent immune activation is believed to play an important role in the pathogenesis of HIV-1 infection. Cellular proliferation can be measured in a number of ways. The gold standard assay to measure CD4 proliferation is the measurement of H^3 -Thymidine incorporation into dividing cells in a short-term culture. More recent methods involve staining cells with Carboxy Fluorescein Succinimidyl Ester (CFSE) which is divided equally between daughter cells during mitosis. Alternately the synthetic nucleoside Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) is an analogue of thymidine and gets incorporated into cells during division. BrdU can be labelled with fluorescent antibodies and used to detect cellular division in culture. Proliferating cells can also be detected by the measurement of surrogate molecules that are upregulated during cell division. These markers have been useful in quantifying proliferating cells *ex vivo*. Protein Ki67 (pKi67) is a nuclear protein which is required for cell proliferation (reviewed in (71)) but its precise function is still poorly understood. It is expressed when cells enter G1 phase of the cell cycle, but is not found in resting cells(203, 204). pKi67 continues to be expressed during G1, S, G2 and M phases, but is highest during DNA synthesis(335). Ki67 staining has been used extensively in HIV-1(29, 108) and

cancer studies(71) to quantify proliferating cells. The protein is expressed in the nucleus and nucleolus, so intracellular staining is necessary for its detection. The transferrin receptor 1 (CD71) also has also been used to identify proliferating cells. Iron is also required for DNA synthesis and progression through the cell cycle (reviewed in(312)). CD71 regulates intracellular iron levels and is required for the uptake of Tf-Fe³⁺ into cells(15, 257). It is undetectable on resting lymphocytes, but is upregulated during their proliferation(138, 536, 543). IL-2 receptor (CD25) expression is required for CD71 expression, and this sequential induction is required for DNA synthesis in lymphocytes(392).

CD57 is expressed on B cells, T cells, NK cells, and NKT cells but its functions are not well defined. The expression of CD57 on CD8 T cells is indicative of extensive proliferation leading to senescence(68) (409). Though cells expressing CD57 are unable to divide, they have been shown to maintain most of their other effector functions, including the secretion of IFN- γ and capacity for cytotoxicity (313). CD57+ cells have been shown to accumulate during HIV-1 and CMV infection (161).

SECTION II. THE HUMAN IMMUNODEFICIENCY VIRUS

Viral classification

The human immunodeficiency virus type-1 (HIV-1) is member of the family of human retroviruses (Retroviridae) (287). HIV-1 is an enveloped virus with a genome that consists of two single strands of sense RNA, of approximately 9.2 kb in size. HIV-1 is classified as a lentivirus because of the long incubation period common before the onset of disease (*lenti* = slow). HIV-1 shares features common to the other lentiviruses. These include the ability to infect non-dividing, terminally differentiated cells, the encoding of regulatory proteins in the viral genome, and a high rate of mutation. All known lentiviruses establish persistent chronic infection by the integration of viral genes into the host genome. Lentiviral infections begin with an acute phase of infection characterised by high viral loads, followed by rapid dissemination of virus and a long period of latent infection, followed by later reactivation accompanied by immunopathology. Other lentiviruses include the simian immunodeficiency virus (SIV), which infects a number of primates, and feline immunodeficiency virus (FIV) which infects cats.

Organisation of the HIV-1 genome

The HIV-1 proviral genome is organised into structural, regulatory and accessory proteins. The structural proteins are composed of Gag, the Gag-Pol precursor, Pro, RT, In, and Env. The regulatory proteins are Tat and Rev, and the accessory proteins are Nef, Vpr, Vpu, and Vif.

Gag

The Gag protein (p55) is a 55kD precursor protein expressed from unspliced viral RNA. Its N-terminus is myristoylated which allows it to associate with the cytoplasmic side of cell membranes(212). Gag recruits viral genomic RNA into budding virions. After budding, the p55 protein is cleaved into 4 smaller fragments, the matrix (p17 MA), capsid (p24 CA), nucleocapsid (p8 NC) and p6. The p17 fragment retains the myristoylated fraction and remains tethered to the inner surface of the membrane to stabilise the virion. It also participates in the escort of the viral DNA to the cell nucleus enabling HIV-1 to infect non-dividing cells (192, 193). P24 forms the conical core of the viral particle. P24 interacts with cyclophilin A (CyA), which facilitates the uncoating of the HIV-1 capsid during the early stages of viral infection (66, 500). NC recognises the HIV-1 packaging signal, to incorporate RNA into virions. This helps to condense viral RNA for packing(532). The p6 polypeptide is involved in the release of budding virions off the cell membrane and aids in the incorporation of Vpr into virions.

Gag-Pol Precursor

The Gag-Pol Precursor protein is formed by differential translation of the viral mRNA. It is generated by a ribosomal frame shift triggered by a cis-acting RNA motif(413). Ribosomes shift to translate the Gag-Pol Precursor approximately 5% of the time, resulting in a 20:1 ratio of Gag to Gag-Pol Precursor. The Precursor is a 160 kD fusion protein containing Protease (Pro, p10), Integrase (In, p31), RNase H (p15), and Reverse Transcriptase (RT, p50) (253).

Pro is an aspartyl protease expressed as a dimer, it cleaves the Gag-Pol precursor, and Gag into the component proteins(31, 179). The protease inhibitors form an important class of drugs active against HIV-1 infection, which work by blocking Pro, resulting in the formation of defective viral particles(127).

RT has both RNA and DNA- dependent polymerase activity, and lacks proofreading activity. The lack of proofreading results in a very high rate of mutation and several point mutations are introduced into each new genome(336, 419). This results in considerable viral diversity that enables the virus to adapt to evade host innate and adaptive immune defences. RT is the target of antiretroviral drugs from the nucleoside analogue RT inhibitor (NRTI) and non-nucleoside RT inhibitor (NNRTI) classes. NRTIs are nucleoside analogues, which get incorporated into the newly synthesised DNA strand. Because they lack a 3' hydroxyl group to form bonds with incoming bases, strand elongation is terminated. 3'-azido-2', 3'-dideoxythymidine (AZT), a thymidine analogue, was the first drug of this type approved for use against HIV-1 infection. NNRTIs inhibit RT by binding at regions other than the nucleoside binding site(120).

The In protein mediates the insertion of viral DNA into the host genome in 3 stages (74, 80). Exonuclease activity trims duplex viral DNA. Double stranded endonuclease activity cleaves the host at the integration site. Ligase activity joins the viral and host DNA. Local host enzymes fill in the ligation site. It is believed that accessibility to DNA rather than specific signal sequences determine the site of proviral integration. Open stretches of DNA that are being actively transcribed are

thus hot spots for integration, and integrating at these sites also increases the likelihood of proviral transcription(437, 483).

Env

The envelope Env protein (gp160) is cleaved into gp41 and gp120. Together gp41 and gp120 are expressed as a trimer forming the virion envelope(43). Gp41 forms the transmembrane domain of Env (307). Gp120 contains the binding site for the HIV-1 receptor CD4. Glycosylation of Env with 25-30 carbohydrate side chains improves binding to the HIV-1 receptor and shields epitopes from neutralising antibodies(569, 589) (440). Gp120 has 5 hypervariable regions, the V3 loop determine whether HIV-1 uses CCR5 or CXCR4 as a co-receptor(129, 173, 245). The V3 loop is also the primary target for neutralising antibodies (nAb); binding this region prevents attachment to the HIV-1 receptor (213). Viruses that bind CCR5 are macrophage-tropic and non-syncytium-inducing (NSI), viruses that utilise the CXCR4 receptor are T cell tropic, and syncytium-inducing (SI). During the course of HIV-1 disease the virus may change tropism and begin to utilise an alternate receptor, the switch from CCR5 to CXCR4 tropism is associated with accelerated disease progression (110).

Tat

The transcriptional activator (Tat) protein is expressed in two forms either 72 or 101 amino acids in length, depending on differential mRNA splicing (459). Both forms activate transcription and bind RNA. Tat binds a stem-loop structure called the transactivation response element (TAR) at the 5' end of viral RNA and activates

transcription from the LTR(172) and increases transcription. Tat binding also promotes elongation at the transcription phase so that full-length transcripts can be synthesised (171, 272).

Rev

Rev (regulator of viral expression) is a 13 kD RNA binding protein that induces transcription. Rev binds the response element (RRE), a bubble formed in the dsRNA helix created by G-G base pairs(37). RRE binding facilitates the export of incompletely spliced RNA and unspliced RNA from the nucleus to the cytoplasm, this mechanism is important in the regulation of HIV-1 late gene expression(342).

Nef

Nef (negative factor) is a 27 kD myristoylated protein. It was originally named negative factor because it was originally thought to down-regulate transcription but it is no longer thought that Nef regulates gene expression. Nef is the first HIV-1 protein detected following infection, this may be one of the mechanisms explaining the immunodominance of Nef responses in many individuals(281). Nef has several functions that increase viral infectivity. Nef down-regulates CD4 expression on the cell surface by interacting with the cytoplasmic tail of CD4, which is subsequently endocytosed and degraded in lysosomes(197, 282). Nef also down-regulates class I MHC expression on the cell membrane, to reduce CTL recognition(484). However Nef does not decrease HLA-C expression, which is a ligand for inhibitory NK cell receptors(14). Nef also increases the expression of FasL on the infected cell, making

it possible to kill approaching CTL that express Fas (590, 597). Nef also activates protein kinases by binding to SH3 domains of Src family tyrosine kinases, allowing it to alter signalling the cell(504).

Vpr

The viral protein, regulatory (Vpr) is homologous to the Vpx protein in SIV. Vpr enables HIV-1 to infect non-dividing cells by tethering the viral genome to the nuclear pore. Vpr also facilitates HIV-1 viral replication by causing arrest in the G2 phase, when LTR are most active(448).

Vpu

The viral protein, unknown (Vpu) is a 16 kD protein which localises at the inside of the target cell membrane. Vpu is necessary for virion budding(286). Vpu is also involved in the decrease of CD4 expression; CD4 and gp120 can adhere to one another in the ER, trapping them inside. Vpu enables separation of the two proteins by triggering ubiquitin-mediated degradation of CD4(579).

Vif

The viral infectivity factor (Vif) inhibits the cellular protein APOBEC3G (498). APOBEC3G is incorporated into HIV-1 virions and is thus transferred to a newly infected cell (16, 525). During HIV-1 reverse transcription in the new cell APOBEC3G causes deamination of cytosine resulting in the accumulation of uracil

bases in the newly synthesised strand of viral DNA (233). The uracils are matched with adenosines resulting in a G → A “hypermutation” of the viral DNA which results in long stretches of adenine bases which interfere with viral viability (258) (598). Vif subverts APOBEC3G function by causing ubiquitination of the protein, thus diverting it to the proteasome for degradation (595).

Regulation of HIV-1 genome expression

The regulation of HIV-1 genome expression is mediated through differential splicing and regulatory proteins. Long terminal repeat sequences (LTR) are positioned at the end of the RNA genome. Transcription from the 5' LTR forms a 9kb transcript that encodes all of the HIV-1 genes. The primary transcript can be spliced into more than 30 different mRNAs or incorporated as genomic RNA into new virions (485). Incompletely spliced RNA encodes the proteins Env, Vif, Vpu, Vpr, and Tat. The fully spliced products encode Rev, Nef and Tat. Rev binds proteins with introns allowing nuclear export of unspliced RNA that would normally be trapped in the nucleus. Threshold levels of Rev are thus required before unspliced or incompletely spliced protein products can be produced. Thus, the “early” HIV-1 proteins are those made from completely spliced RNA (Rev, Nef, Tat) and are Rev-independent. The “late” genes require Rev production for their expression (Gag, Pol, Env, Vpr, Vpu, Vif).

The LTRs sequences contain 3 sub-regions that regulate HIV-1 transcription, U3, R and U5. The LTR contain cis-acting DNA sequences for binding of cellular transcription factors. These includes binding sites for cellular transcription factors NF-κB, SP-1, Ets, NF-AT and AP-1 (389). Binding of these DNA sequences

enhances HIV-1 transcription. The LTR also contains the TAR and the site of initiation for transcription.

The HIV-1 life cycle

The life cycle of HIV-1 begins with viral binding and entry. Env fuses with the host cell membrane at CD4, inducing a conformational change in gp120 that exposes the co-receptor binding site for CCR5 or CXCR4(303). Binding induces a conformational change in gp41, bringing together the HR1 and HR2 regions of the protein to form a 6-helix bundle that draws the virion and cell together forming the fusion pore (91, 348, 364). Following fusion the contents of the virion are released into the target cell. A class of ARVs called fusion inhibitors has been developed to block viral fusion (344). HIV-1 can also be endocytosed but this does not result in productive infection and usually results in lysosomal degradation (347). In the cytoplasm CyA, Nef and Vif participate in the uncoating of the virion associated with the plasma membrane. Reverse transcription occurs in the cytoplasm to create double stranded viral DNA, tRNA^{Lys} acts as a primer for RT. RNase H cleaves RNA-DNA hybrids, removing the reverse-transcribed strand to allow synthesis of complementary DNA. HIV-1 proteins and the viral dsDNA form the pre-integration complex (PIC), which is transported to the nucleus. The viral genome is compacted in the PIC and crosses the nuclear membrane with the aid of In, MA, and Vpr. Integration of viral DNA into the host chromosome is mediated by In, which cuts both viral DNA, host chromosome, and then joins the two together. Integration can lead to active or latent infection of the cell. Proviral DNA not being actively transcribed can persist in cells as a viral reservoir. Viral latency makes complete eradication of the virus unlikely using antiretrovirals. Engagement of transcription factors at the LTR initiates proviral

transcription and is greatly enhanced by the binding of Tat to TAR. Viral transcripts are generated and transported to the cytoplasm in a Rev-dependent or –independent manner. Virion assembly occurs adjacent to the plasma membrane. MA anchors the assembling complex to the membrane. Gag, Gag-pol, Vpr, and genomic RNA are packaged into the virion by the co-operation of viral and cellular chaperone proteins. Budding occurs through hijacking of the cellular export machinery. The p6 protein binds the cellular protein tumour-suppressor gene 101 (TSG101) and exits the cell by budding in a way similar to the formation of exosomes (209).

The early history and epidemiology of HIV-1

The first cases of AIDS were identified in the early 1980s when doctors from New York, Los Angeles and San Francisco reported cases of *Pneumocystis jirovecii* pneumonia (formerly *P. carinii*) (PCP) and Kaposi's sarcoma (KS) in young homosexual men and injection drug users (3, 7, 352). Because the first cases were identified amongst homosexual men, the condition was for a time called Gay-related immune deficiency syndrome (GRIDS). Soon there were similar reports in injecting drug users and haemophiliacs, and Haitians with no reported homosexual behaviour (8, 352). All of the affected subjects had severely depleted CD4 counts. The first reports of AIDS in an infant was in the setting of blood transfusion (494), but it soon became clear that the infectious agent could be transmitted vertically (486) (114) (515) (97) (329). Together these case reports suggested the infectious agent was a blood-borne pathogen that could also be transmitted sexually. In 1982 the CDC adopted the name acquired immune deficiency syndrome (AIDS) and published its first case definition (9). The virus responsible for AIDS was first isolated in 1983 by Françoise Barré-Sinoussi and was called lymphoid associated virus (LAV) (36), but it

was not known at the time to be responsible for AIDS. In 1984 two laboratories independently published reports identifying the virus causing AIDS, and these were called HTLV-III (human T lymphotropic virus) and AIDS associated retrovirus (ARV), respectively (194, 322). In 1986 the International Committee on the Taxonomy of Viruses established the name human immunodeficiency virus (HIV). HIV-2 was discovered in 1986, and the original virus was renamed HIV-1 (100, 224, 270).

In Europe and North America, rates of HIV-1 infection continued to climb in the 1980s and early 1990s, particularly in high-risk groups of homosexual men, injection drug users and commercial sex workers. The screening of blood transfusion products, public health awareness, and risk reduction in high risk groups greatly contributed to control of the epidemic in resource-rich nations. Treatment of HIV-1 with multiple antiretroviral drugs began in 1996, (highly active antiretroviral therapy, or HAART). HAART is able to reduce levels of viraemia to undetectable levels, but the presence of a reservoir of latent provirus makes it unlikely that HAART will ever clear virus completely (586) (98) (178). HAART greatly increased survival in people living with HIV-1, and dramatically reduced the number of AIDS cases and opportunistic infections (2). Though HIV-1 was first identified in the US and Europe in the 1980s, it soon became clear that the virus was already well established in sub-Saharan Africa (471, 551) (296). Over the next 20 years HIV-1 prevalence continued to grow in sub-Saharan Africa and this region continues to carry the highest prevalence.

In sub-Saharan Africa, HIV-1 infection occurs predominantly through heterosexual and mother-to-child transmission (MTCT). The latest WHO/UNAIDS Epidemic

estimates 39.5 million people are infected with HIV-1 globally, with 24.7 million in sub-Saharan Africa (547). Globally, 2.3 million children under the age of 15, and 18 million women are infected with HIV-1. There were 4.3 million new infections in 2006, and 530,000 of these occurred in children under the age of 15. Overall, 2.9 million deaths were attributed to HIV-1 and 380,000 of these were among children. At the 2005 G8 Summit and the September 2005 United Nations World Summit, the leaders of the world's nations committed to a massive increase in HIV-1 prevention, treatment and care. A goal was set to provide universal access to HIV-1 treatment for all who need it (575, 576). Several governments, non-governmental organisations, and private charities have donated billions of dollars and drugs for HIV-1 treatment in less developed countries. However, poor health care infrastructures and the lack of experienced health professionals are major hurdles to meeting this goal. Stigma continues to be a barrier to HIV-1 testing and partner notification, many HIV-1 infected people fail to access public services because they do not yet know they are infected.

Origins and diversity of HIV

HIV-1 sequencing and phylogenetics have traced the likely origins back to sub-Saharan Africa. HIV-1 is most similar to the simian immunodeficiency virus (SIV) virus found in chimpanzees (196), and HIV-2 appears to be closely related to SIV isolated from sooty mangabeys (95) (225). HIV-2 is endemic in West Africa and regions once colonised by Portugal. The earliest confirmed HIV-1 specimen was isolated from a plasma sample taken from a man in the Democratic Republic of Congo in 1959 (600). Phylogenetic analysis of the viral sequence placed the specimen nearest to the ancestral nodes of B and D subtypes. Based on sequence

variation and phylogenetic relationships of different subtypes, the jump from chimpanzees into human beings probably occurred in central Africa around the 1930s (± 10 years) (289) (465) (555).

HIV-1 is organised into subtypes based on sequence similarity. HIV-1 is organised into 3 main groups, M, which accounts for the majority of viruses circulating in the global pandemic, O, which is found in low prevalence in west and central Africa and N which is rare and found only in Africa. The M group is divided further into subtypes (also called clades) A-K (there are no E and I subtypes), and several circulating recombinant forms (CRFs) that comprise viruses clearly originating from the recombination of two subtypes. Subtypes A and F are further divided into A1, A2, F1 and F2. Sub-Saharan Africa contains the most diverse collection of subtypes, A, C, D and CRF02_AG. Subtype B is responsible for the epidemics in Europe, Australia, and the Americas, though other subtypes are becoming more frequent. Subtype C is the most common in southern Africa and India, and is accountable for nearly half of infections globally. Subtype A (~70%) and D (~21%) are the most frequently isolated in Kenya and intersubtype recombinants comprise at least 2% of infections (393).

HIV-2

HIV-2 shares 30-60% homology with HIV-1, and utilises the same co-receptors and target cells as HIV-1. HIV-2 infection also leads to AIDS, but long-term survival is more common (271, 349, 351). HIV-2 is also transmitted vertically, though less frequently than HIV-1, and is associated with increased risk of mortality compared to

HIV-negative children(476). HIV-2 patients in chronic infection typically have proviral loads comparable to HIV-1 infection, but plasma viral load is much lower than observed in HIV-1, even in the absence of therapy. CD8 T cell responses of similar magnitude are detected against HIV-1 and HIV-2. However, CD4 T cell proliferation and cytokine production appear to be much stronger against HIV-2 (153), and NK cell activity may be greater (397). Early reports suggested HIV-2 may confer protection from HIV-1 infection, but this has not been confirmed in subsequent studies. Dual infection is common and results in disease progression similar to that observed in HIV-1 infected individuals (475).

Transmission of HIV-1

Transmission of both HIV-1 and HIV-2 occurs through the transfer of free virions or infected cells across mucosal surfaces. HIV-1 is also found in blood, semen (297, 368), vaginal and cervical secretions (295) (101), breast milk (391) (454) and amniotic fluid (384). The likelihood of HIV-1 transmission is influenced by a number of factors. Transmission is most likely to occur if the infecting person has a high viral load, particularly in acute infection (176) (137). The presence of STDs, and genital ulcers in particular increases the risk of HIV-1 acquisition (83) (503).

Though the person-to-person risk for sexual transmission of HIV-1 is low (0.1% - 0.4% for sexual transmission) (217, 458) the risk of MTCT is very high, with estimates between 25-40% in breast-feeding populations (390) (11). MTCT of HIV-1 can occur *in utero*, during delivery, or through the ingestion of breast-milk. It is not always possible to discriminate the timing of transmission, but it is thought that most infections occur *intrapartum* through exposure to maternal cervical and vaginal

secretions and/or blood(122, 155). Maternal plasma viral load has consistently been correlated with the risk of MTCT (137) (375) (513). Women with detectable HIV-1 shedding in the genital tract are also more likely to transmit virus to their infants (263). Chorioamnionitis, prolonged rupture of membranes, and genital tract infections are also associated with increased risk of transmission (528) (516) (374). Caesarean section reduces the risk of HIV-1 transmission by preventing exposure to the maternal cervical mucosa, but carries additional risks for the mother(343).

Acquisition of HIV-1 *in utero* is also common. HIV-1 has been detected in amniotic fluid, (384) the placental trophoblast, and Hofbauer cells (a macrophage) of the chorionic villous(92, 324, 367). However placental infection does not correlate with transmission, and HIV-1 replicates poorly in trophoblast cells *in vitro*(33, 350, 354, 376). The placenta appears to be a fairly good barrier against MTCT, and transmission probably occurs via transcytosis of cell-associated virus (304) or perhaps through the use of DC-SIGN (509). Though infection can happen as early as 8 weeks in gestation(324), most transmissions occur late in gestation, within a month of delivery(70, 455).

Breast-feeding poses an additional risk of infection to the infant, especially during the first month of life when the breast milk contains high levels of cell-associated virus(390, 391, 552). Risk of acquisition after the first month is lower but constant over time and related to the duration of breastfeeding(113). Viral load in the breast milk correlates with plasma viral load, but is approximately 2-3 logs lower(454). Breast milk contains epithelial cells, lymphocytes and macrophages that can all be carriers of HIV-1. It is thought that transmission via breast-milk occurs across both

the oral mucosa(187, 188) and the gut mucosa (23) (56). Levels of maternal plasma and breast milk viraemia are strong predictors of transmission (317). Mastitis and sub-clinical mastitis during lactation were shown to further increase the risk of HIV-1 transmission by increasing breast milk HIV-1 viral load (490) (580).

A number of interventions have been shown to reduce the risk of MTCT. The overall strategy of these interventions is to reduce the exposure to maternal virus. If an infant escapes transmission *in utero* and during delivery, breast milk transmission can be eliminated by replacement feeding. However, in most African nations breastfeeding can be nearly ubiquitous for various cultural, economic, and nutritional reasons. The choice not to breastfeed is essentially a revelation of one's HIV-1 status to family and community. Furthermore, breastfeeding is important to the health of the infant, and its benefits must be weighed against the risks imposed by HIV-1. In a Kenyan study, formula-fed infants not acquiring HIV-1 had rates of mortality comparable to those with HIV-1(390). In addition to the nutritional benefits, neonates acquire all of their IgA from breast milk, and additionally benefit from maternal IgG. The preparation of safe breast milk substitutes makes replacement feeding dangerous for women without access to clean water, increasing the chance of infection with water-borne pathogens. Several studies have shown that exclusive breastfeeding reduces the risk of HIV-1 acquisition compared to mixed feeding (112, 247). In many communities, giving the infant water and soft foods is begun within the first few weeks of life. The premature introduction of food and water-borne pathogens may initiate inflammatory reactions in the neonatal gut, thereby creating a mucosal environment facilitating HIV-1 entry(449). The WHO thus recommends avoidance of breast-feeding only where it is acceptable, feasible, affordable, sustainable and safe. If the mother is known to be

HIV-1 infected it is recommended that exclusive breastfeeding continue until 6 months, followed by re-evaluation of the risks and benefits with continued breast feeding(577).

The provision of antiretrovirals (ARVs) to the mother and/or infant greatly reduces the risk of MTCT. The earliest intervention trials used AZT monotherapy initiated between 14-34 weeks of pregnancy and provided a high intravenous dose during delivery, and AZT treatment to the infant for 6 weeks post partum (109). The intervention reduced the risk of transmission by ~70%. A subsequent study in Thailand showed that short course regimens during pregnancy in the mother alone significantly reduced risk of *in utero* and *intrapartum* transmission(492). The current gold-standard for prevention of MTCT is HAART during pregnancy, followed by the avoidance of breastfeeding. Elective Caesarean may be beneficial if complete viral suppression cannot be achieved in the mother. Together, these interventions have reduced the risk of transmission to < 2% in resource rich settings(111). Current WHO guidelines recommend a two-part approach for ARV prophylaxis in resource-poor settings. If the mother requires treatment for herself, the WHO recommends use a nevirapine (NVP) based combination therapy plus prophylaxis for the infant. If the mother does not require treatment for her own health the WHO recommends short-course combination therapy plus single-dose *intrapartum* and neonatal NVP (117) (575). Though these interventions greatly reduce the risk of MTCT, these interventions are still unavailable to most women in resource-poor settings. In Kenya, where HIV-1 prevalence among pregnant women is currently estimated to be 6.1%, only 9.3% currently benefit from interventions to prevent MTCT.

The natural history of HIV-1 infection

HIV-1 infection in adults

Acute infection with HIV-1 is often asymptomatic, though a proportion of people experience a seroconversion illness characterised by flu-like symptoms resembling infectious mononucleosis. During this time virus is disseminated from the site of infection to the lymphoid tissues. Viraemia peaks at about 3 to 4 weeks post infection and is followed by a period of decline and it is thought HIV-1 specific CD8 T cell responses are responsible for this reduction in viral load(292, 402, 560). Antibodies to HIV-1 are generated 2-3 months post-infection, resulting in a “window period” when the newly infected person may be infectious but still seronegative. CD4 cells drop rapidly during acute infection, and then partially recover their numbers as viral levels decline. It is now known that the majority of CD4 depletion occurs in the gut associated lymphoid tissues (GALT) and persists here even following the partial recovery observed in the blood (69).

Following acute infection, an equilibrium is reached between host and virus in which relatively low levels of viraemia are maintained for many years in most individuals before the onset of AIDS (411). In the absence of ARV therapy, mean time to AIDS is approximately 8-10 years in adults, though this is influenced by age, and may be significantly shorted in resource poor settings (256, 460). During this time the host is usually asymptomatic, though viral replication continues, and CD4 T cells are steadily depleted. Levels of viraemia in early infection are highly predictive of the rate of disease progression (310, 366, 399, 521). Lower viral loads during acute infection result in more limited destruction of the CD4 subset and more slowly progressive

disease (162, 379, 431). CD4 T cells are progressively depleted during the asymptomatic period, until a critical threshold is reached. The loss of CD4 help disables the immune response allowing the invasion of opportunistic pathogens, the onset of AIDS, and ultimately death.

Many of the infectious organisms causing severe disease in immunosuppressed patients are present in immunocompetent people without causing disease, hence the pathogens cause “opportunistic infection” (OI) (353). OIs vary greatly by environmental exposure to pathogens and CD4 count. *Mycobacterium tuberculosis* (TB), KS, PCP, CMV, toxoplasmosis, and B cell lymphomas associated with Epstein-Barr virus (EBV) infection are common globally. OIs may also disseminate beyond their usual reservoirs to cause systemic disease or pathology in multiple organs. A good example is CMV, which asymptomatically infects most of the world’s population. In immunosuppressed patients CMV becomes reactivated, and causes symptomatic disease. In HIV-1 infection CMV can cause severe retinopathy, gastrointestinal disease, and infection in the central nervous system.

T cell responses in HIV-1 infection

There is a wealth of evidence supporting the importance of CD8 T cells in the control of HIV-1 replication. HIV-1- specific CTL appear during acute infection prior to the emergence of HIV-1 antibodies, peak with viral load, then inversely correlate with viral load during post-acute infection (292, 387, 402). Blocking of CTL with antibodies during acute SIV infection results in uncontrolled viraemia (481) (261). The importance of CTL is further supported by the emergence of “CTL escape variants,” mutations in the HIV-1 genome that affect antigen processing, MHC

binding, or TCR recognition (62, 292, 315) (150, 421, 435). The emergence of escape has in some cases been associated with increased viral replication and disease progression (215). Two selective forces thus act upon the virus, the need to maintain replicative fitness and to escape CTL(250, 319). The detection of HIV-1 specific CD4 proliferation has also been strongly correlated with slow disease progression in HIV-1, though in the majority of HIV-1 infected subjects, these responses are low or undetectable(268, 388, 450). Despite the low frequency of proliferative responses, HIV-1 specific CD4 T cells can be detected in many individuals by the secretion of cytokines (IFN- γ , IL-2), and their frequencies decline with time during chronic infection (426).

The characterisation of subjects with slowly progressive disease suggests that the “quality” of the T cells generated during the adaptive immune response is an important factor in the control of viraemia. During chronic HIV-1 infection, the frequency of HIV-1 specific CD8 measured by IFN- γ are not consistently correlated with viral load and survival(10, 44, 154, 201). Long-term survivors have been shown to generate CTL with multiple effector functions in addition to cytotoxicity, including the secretion of one or more cytokines and proliferation (45) (19). The maintenance of CD4 help is important for the sustained function of CTL during chronic infection (326) (229). CD4 help may be vital to the generation of functional effector cells. In the mouse LCMV model, virus-specific CTL can be generated in the absence of CD4 T cells, but lack the ability to secrete cytokines or to kill(596). CD4 help appears to be maintained in HIV-2 patients who typically experience slow disease progression(153).

There is also accumulating evidence of that the lack of effector function of CTL during chronic HIV-1 infection is due to “exhaustion”. HIV-1 specific CD8 T cells acquire CD57⁺ expression, indicative of senescence (68). In addition to decreased proliferative capacity, there is good evidence for the progressive loss of other CTL functions during disease progression (27) (493) (290). The recent identification of the cell surface molecule programmed-death-1 (PD-1) on HIV-1 specific CD8 T cells is associated with an exhausted phenotype, characterised by a lack of cytokine secretion, killing, and proliferation (542). In a cohort of HIV-1 infected patients, frequencies of PD-1⁺ cells and the level of PD-1 expression were strongly correlated with HIV-1 viral load and progression (119). The blocking of PD-1 with antibody was shown to reverse the effects and restore effector function, and this has generated interest in the therapeutic possibilities of targeting the molecule.

Mechanisms of CD4 depletion

CD4 T cells are the main targets of HIV-1 infection, however macrophages may also be productively infected and may be important reservoirs in the tissues. Despite the rapid and progressive depletion of CD4 T cells, it is now clear that only a subset is actually infected, and a large number of cells die thorough mechanisms other than direct cytopathic effects of the virus. The progressive depletion of T cells is thought to progress by a combination of 1) direct cytopathic effects on infected cells 2) killing of innocent bystander cells 3) impaired CD4 proliferation (in infected and uninfected T cells) 4) dysregulation of haemopoiesis and production of naïve cells.

Proliferation is impaired in HIV-1 infected CD4 T cells, and they are more likely to undergo apoptosis than uninfected cells (235, 415, 501, 585). CD4 activation is not

impaired, and a number of infected cells are also depleted by AICD. Memory cells, and HIV-1 specific CD4 T cells in particular are preferentially targeted for HIV-1 infection, further reducing the pool of effector cells available to fight infection (145). The expression of HIV-1 viral proteins Tat, Nef, and Vpr can also induce apoptosis (573). Depletion of CD4 occurs primarily in bystander cells which are not infected with virus (177, 386). Bystander cells can be eliminated by the formation of syncytia (240, 470), AICD, and the induction of apoptosis by cells bearing FasL(17, 142) (64).

T cell haemopoiesis is also dysregulated by HIV-1 infection. Naïve CD4 and CD8 T cells are also depleted during the course of HIV-1 (447). Signal-joint TCR excision circles (TREC) generated during TCR rearrangements are diluted with successive cellular divisions, and can be used to monitor the relative proliferation of T cells. HIV-1 infected subjects have lower levels of TRECs in comparison to HIV-1 negative age-matched controls, and had lower frequencies of recent thymic emigrants (RTEs), suggesting impaired thymic function (148). Treatment with HAART increased TREC levels and the frequency of naïve T cells. CD34+ progenitor cells have also been found to be infected with HIV-1 (345, 520) and express high levels of CXCR4, suggesting HIV-1 infection may also have the potential to disrupt haemopoiesis (128).

Host genetic factors influencing the control of HIV-1

Host genetic factors have been shown to contribute significantly to the risk of HIV-1 acquisition and disease progression. Mutations in chemokine receptor genes have also been shown to influence the risk of HIV-1 disease progression. A 32 bp deletion in the CCR5 exon (CCR5 Δ 32) results in a lack of CCR5 expression on the cell surface (331) (466). Subjects homozygous for this mutation are unable to be infected

by strains with R5 tropism. Subjects heterozygous for the mutation can be infected with R5-tropic viruses but infection is less likely than in the absence of the mutation. However, the cells of these patients can still be infected with X4-tropic HIV. Additional mutations in the chemokine receptor genes have also been identified that provide reduced risk of HIV-1 infection or disease progression: 59356C/T CCR5 promoter, CCR2-64I, SDF-1 3'A, and RANTES (25, 262, 264, 330, 523). The possession of several class I HLA molecules has been associated with the risk of HIV-1 acquisition or disease progression (86). Homozygosity for class I alleles has also been associated with bad outcome (85), but homozygosity for HLA-Bw4 appears to be protective (180). In MTCT, the sharing of more than 3 class I HLA alleles has also been shown to increase the risk of MTCT (340, 433).

Paediatric HIV-1 infection

Paediatric HIV-1 infection results in an accelerated course of disease compared to adults. In untreated infants, viral loads are maintained at very high levels during the first two years of life, and at this time there are high rates of mortality (382, 496) (410). US and European cohorts have reported 2 year mortalities of ~2-20% (50) (216) (6). Studies from sub-Saharan Africa have reported much higher rates of mortality (395). A study in West Africa reported 60% mortality at 18 months (116), and studies from East Africa have reported 20%-52% (355, 401) (160). The greatest risk of AIDS appears to be during the first year of life, those children surviving the first year are able to establish control of virus and survive even in the absence of ARV (50) (216) (134). This observation suggests strong selection factors during the first few months of life may influence survival.

Correlates of mortality

As in adult infection, HIV-1 viral load during early infection is a strong indicator of disease progression and mortality in infants (136) (453) (443) (67). Various studies have used mean, peak, or baseline viral load to predict survival. *In utero* transmission and early *peri partum* infection have been associated with increased morbidity and mortality in MTCT (318) (355). Maternal health is also a very strong predictor of infant survival, particularly during the first year of life, whether or not the infant becomes infected with HIV-1. Maternal HIV-1 viral load, CD4, and mortality have been shown to predict infant mortality (249) (401) (443). Breastfeeding, though a risk factor for HIV-1 infection, is important in infant health and the prevention of respiratory and diarrhoeal illnesses in both HIV-1 infected and uninfected children (1, 495).

The most common causes of death during the first year of life in HIV-1 infected infants in resource-poor settings are diarrhoeal and respiratory disease. Repeated episodes of infection with *Candida albicans*, oral hairy leucoplakia, and pneumonias (often PCP) and non-specific gastrointestinal illness are very common in HIV-1 infected infants.

Treatment

Though a staggering number of children are currently infected with HIV-1, there is a lack of data informing treatment guidelines. Because untreated HIV-1 is known to follow such an aggressive course in infants, in resource-rich nations it is now standard to treat children immediately following confirmation of HIV-1, regardless of CD4 or viral load. However, the optimal regimen for neonates, and whether treatment can be

safely discontinued at an older age is unknown. In sub-Saharan Africa, these problems are especially pressing due to the great numbers of infected children. Additionally, rural and resource-poor areas may lack diagnostic facilities to identify HIV-1 in infants. Subsequently, many areas rely on clinical indicators to diagnose HIV-1 infection in infants. Many ARVs are not provided in paediatric formulations, those that are available as liquids require refrigeration, and this is a problem if patients do not have electricity. A recent report by the CHER study group in South Africa has demonstrated that ARV therapy initiated in the first 12 weeks of life can substantially reduce morbidity and mortality during the first year of life (558). The frequency of treatment failure and severe adverse effects were low in the infants and treatment compliance was very high. This report suggests that the treatment of very young infants with HIV-1 is viable and beneficial. Several other clinical studies are currently underway in sub-Saharan Africa to optimise HAART in children (166) (398) (452) (561). Several medical interventions besides HAART have also been shown to benefit infants with HIV-1 infection. The WHO recommends all children with suspected HIV-1 infection be given trimethoprim sulphamethoxazole (TMP-SMX) prophylaxis for the prevention of *Pneumocystis jiroveci* pneumonia (PCP) (88). Vitamin A supplementation is also recommended for the reduction of infant morbidity and mortality (168).

Immune responses during paediatric HIV-1 infection

Innate immune responses

NK responses to HIV-1 have been demonstrated in HIV-1 and HIV-2 infection (397). Increased NK activity and release of IFN- γ has been correlated with HIV-1 infection, though their overall frequency may be reduced (21). NK cells release β -chemokines MIP-1 α , MIP-1 β and RANTES that compete for the CCR5 receptor and thus have the ability to block HIV-1 entry into target cells(405). High levels of chemokines in breast milk have been associated with reduced risk of MTCT(163). ADCC antibodies directed against HIV-1 have been identified in adult and infants with HIV-1 infection. IgG ADCC antibodies are obtained through placental transfer, but have not been associated with protection from transmission(259). Furthermore although ADCC antibodies could be detected in infants, these were not associated with disease progression, and children less than a year old were unable to make ADCC responses against HIV-1(259). CD8 antiviral factor (CAF) activity has also been associated with delayed disease progression and has also been detected in infants exposed to HIV-who escaped infection(323). Secretory leukocyte protease inhibitor (SLPI) is a mucin that may prevent HIV-1 entry, SLPI levels in saliva and vaginal fluids have been shown to correlate with reduced transmission(165, 424), but may not protect from breast milk transmission.

Humoral immune responses

Overall HIV-1 specific IgG titres correlate poorly with transmission. Neutralising antibodies (nAb) correlate better with protection from transmission than overall IgG

titres and have been promising in animal vaccine models(135, 226, 285, 472, 554). IgA nAb against HIV-1 have also been found in HIV-1 infected patients and in exposed seronegatives (ESN) (102, 132). In MTCT viral species that have escaped maternal antibody responses are preferentially transmitted to the infants, suggesting that selective pressure from infant nAb is involved in the selection of transmitted variants(588). However, the sites of Env which contain nAb epitopes are easily mutated by HIV-1. Glycosylation of gp120 results in further shielding of neutralising epitopes(569). The search for antibodies that can broadly neutralise many primary viral isolates has produced few strong candidates.

T cell responses to HIV-1

HIV-1-specific T cell responses are less frequently detected in neonates and infants(338, 339) (96, 337) (423) (76) (487) (333) (506) (534). In contrast, infection with CMV results in the expansion of very high frequencies of CMV-specific CTL that have a mature phenotype and function similar to those observed in adults (94) (346) (206, 372). The CD4 response to CMV however, appears to be less than fully functional than it is in adults with lower production of IFN- γ (87) (546). These responses are sufficient to enable most children acquiring CMV *post partum* to remain asymptomatic, although viral shedding may persist for years (265) (13) (517). In contrast CTL responses from neonates and infants fail to control HIV-1. In most infants viral loads are maintained at persistently high levels for at least a year, and mortality is very high during the first two years of life(382, 410, 496).

In general, infant HIV-1-specific T cell responses resemble those generated by adults with rapid progression. Infant CD8 T cell responses against HIV-1, measured by cytotoxicity assays or cytokine production, are less frequently detected and weaker in magnitude than responses detected in adults (338, 339) (337) (423) (76) (487) (333) (506) (534). The frequency of IFN- γ producing cells has not been shown to be associated with viral load or disease progression in infants, though in older children there are reports of positive and negative associations with disease progression (77, 79, 333, 446, 489). Early treatment with HAART has been shown to decrease the magnitude and breadth of IFN- γ responses in children compared those treated later in life, and detection of HIV-1 specific CD8 is associated with incompletely suppressed viraemia (61, 78). Env-specific IFN- γ responses are more frequently detected in infants compared to older children(76, 534).

As observed in adult infection, CD4 responses, including proliferation(370) and cytokine production, appear to be particularly impaired in paediatric HIV-1 infection (298, 564). The maintenance of Th1 responses has been associated with sustained CTL responses and slow disease progression in children with HIV-1(90, 301, 534, 566). Infant CD4 responses may be preferentially skewed toward a Th2-type response in CMV and HIV-1 infection, which is not optimal for antiviral responses (434, 546, 564). However, infection *Bordetella pertussis* or pertussis vaccination results in the generation of Th1 type responses in neonates (462) (461). In those children surviving the first year months of life, T cell responses grow in frequency and magnitude(78, 333, 467).

Factors contributing to impaired HIV-1 responses in infants

Many factors may contribute to lower T cell responses in infants. Several studies have reported Th-2 skewed responses, or weak IFN- γ production in CD4 T cells during viral infection. IFN- γ responses may be suppressed in the foetus to prevent placental damage (568). These responses continue to be impaired during neonatal life, and are observed as a skewing toward a Th2 type T cell response to viral infections (434). Down-regulation of IFN- γ synthesis is thought result from hypermethylation of CpG sites within and adjacent to the IFN- γ promoter (574). Hypermethylation of the IFN- γ gene was observed in CD4+CD45RO- when compared to adults. However methylation of the IFN- γ gene of CD8+CD45RO- and NK cells were similar to that observed in adults, and corresponded to similar levels of IFN- γ production. Methylation of other cytokines promoters IL-4 and TNF- α were similar between neonatal blood and adult DNA, suggesting a selective mechanism for down-regulation of foetal IFN- γ in CD4 cells. This selective regulation of IFN- γ may explain the reduced Th1 responses observed during HIV-1 and CMV infection in neonates.

Additionally, HIV-1 has been shown to infect the thymus (70), and HIV-1 induced destruction of developing lymphocytes may affect T cell responses. *In vitro*, human and mouse neonatal T cells are less responsive to stimulation through the TCR. Cord blood and neonatal CD4 T cells require more co-stimulation in order to produce IFN- γ and IL-2 and to proliferate, but the provision of adult APC and co-stimulation can generate adult-like responses(12). Neonatal T cells have lower expression of TCRs and adhesion molecules LFA-1 (CD11a), LFA-3(CD58), and CD2 (468) (278), which

could result in lower signalling through the TCR, and a greater need for co-stimulation.

Genetic similarity of mother and infant may result in the transmission of virus pre-adapted for efficient replication in the new host. CTL escape mutations have been detected in infants(214). *De novo* CTL escape mutations have also been detected in infants (170) (425) suggesting that although CTL responses may be weaker during infant life, they have sufficient function to exert selection pressure on the virus to mutate.

Exposed uninfected individuals

HIV-1 specific CD4 and CD8 T cell responses have been detected in subjects with persistent exposure to virus who remain uninfected. These groups include commercial sex-workers (CSW) (183 {Kaul, 2001 #49, 184, 275, 276, 456), discordant couples (223) and infants born to infected mothers(105, 299, 300, 316, 457). Long-term follow-up of CSW suggests that these responses may be indeed protective and not just an effect of exposure(277). The mechanism underlying the generation of these responses in the absence of infection is not known. Cross-presentation of defective virions, viral proteins, or apoptotic cells by DCs may explain CTL responses in the absence of infection. Occult infection may also explain some of these cases, recent ultra-sensitive PCR has revealed several exposed seronegatives to be infected with virus at very low levels (599).

Development of an HIV-1 vaccine

The development of a safe, effective, affordable vaccine against HIV-1 is the best hope for containing or ending the pandemic. Initial strategies focused on generating nAb responses to the HIV-1 Env, but these have failed to protect in phase III trials (181, 427). The flexibility of the viral envelope in subverting nAb responses via mutation and glycosylation suggest this may not be effective as a single strategy (445). The strong evidence supporting the protective role of CTL in prevention of HIV-1 acquisition and delayed disease progression has focused much of the current research on the development of a vaccine to induce cellular immune responses against HIV-1. An optimal vaccine may indeed elicit both humoral and cellular responses. Viral diversity has been the main challenge in the design of a vaccine. Heterogeneity between different viral subtypes may necessitate the development of subtype-specific or vaccines. Alternatively a polyvalent vaccine may be optimal for regions with multiple subtypes, such as Kenya where subtypes A and D are circulating at high prevalence(393). In addition to viral heterogeneity, host HLA alleles differ greatly between different regions and this must also be considered in evaluating immunogenicity.

Safety issues have precluded the development of inactivated or live attenuated HIV-1 as a vaccine. A strategy adopted by several groups is the inclusion of HIV-1 genes in a viral vector with limited replicative ability, such as vaccinia, adenovirus, Semliki forest virus or canarypox. These have been shown to be immunogenic alone or combined in a two-step prime-boost with a DNA-vector. A novel approach has been to include HIV-1 genes in a *Bacillus Calmette Guérin* (BCG) for the simultaneous

immunisation of neonates against HIV-1 and TB (248). Choice of HIV-1 genes for inclusion will also be important to immunogenicity; it may be best to target the most abundantly expressed structural proteins (Gag, Env), or regulatory genes expressed early (Rev, Nef, Tat). Another strategy has been to select individual epitopes targeted by resistant individuals (exposed seronegatives) on the assumption that these responses may confer protection. The use of adjuvants and different routes of inoculation can also greatly improve immunogenicity.

Though a prophylactic vaccine is the holy grail of vaccine design, sterilising immunity may not be possible in the absence of an antibody component. However, several vaccine/challenge experiments in primate models indicate that a vaccine might improve survival even if it cannot confer protection from infection. Such a vaccine may limit CD4 destruction by reducing viraemia during acute infection, resulting in better long-term containment of viraemia (320). Additionally, a therapeutic vaccine would be of great benefit to those who are already infected. This strategy focuses on vaccination to enhance the immune responses of those already infected with the virus, in order to enable better long-term viral containment and possible cessation of antiretroviral therapy (143) (144).

SECTION III. CYTOMEGALOVIRUS AND HIV-1

Classification and epidemiology

Cytomegalovirus (CMV) is a linear double-stranded DNA virus from the betaherpesvirus family (human herpesvirus-5, HHV-5). The receptor and co-receptor for CMV have recently been identified as epidermal growth factor receptor (EGFR) and integrin $\alpha v\beta 3$ (562, 563). CMV infects a wide array of cell types including endothelial cells, neutrophils, and lymphocytes (287). Its main reservoir in the blood is the monocyte. CMV infects the majority of the world's population and prevalence is higher in areas of low economic development (195).

CMV is the leading cause of congenital viral disease globally, affecting approximately 0.1-2% of all births (185) (200) (479). In developing nations, CMV may infect a large percentage of children during the first year of life (40). CMV is transmitted through contact with infected secretions, the incubation period is 2-3 weeks after which virus can be detected in blood, saliva, genital secretions, urine, and breast milk (559) (227) (517). Most symptomatic congenital infections occur in settings where the mother is experiencing a primary CMV infection. Transmission is less likely during latent maternal infection and the majority of these are asymptomatic (58, 186). Symptomatic CMV is more likely in the setting of detectable CMV viraemia (306). Symptoms of congenital CMV are both immediate and long term. Immediate symptoms may include hepatomegaly, splenomegaly, microcephaly, jaundice, thrombocytopenia or petechial rash. Long-term effects of congenital CMV include deafness, vision impairment, mental and growth retardation (35, 57, 414).

Chronic infection and immunosuppression

Though the immune system effectively contains CMV replication, persistent latent infection is established for the life of the host. Most healthy people will never become symptomatic, but there is a growing body of evidence suggesting that long term infection with CMV may compromise immune responses in the very old (334, 406) (417). CMV may become reactivated during treatment with immunosuppressive drugs or during HIV-1 infection. CMV infection causes disease in different organs depending upon the underlying basis of immunosuppression(158). CMV in transplant recipients may cause fever, hepatitis, gastrointestinal tract inflammation (GI), retinitis, pneumonitis and myelosuppression. In HIV-1 infected adults, CMV retinitis is the most common syndrome associated with CMV disease, but dissemination to the GI and central nervous system tissues is also common. HIV-1 infected infants with congenital CMV may have GI disease and failure to thrive. HAART has greatly reduced CMV-associated morbidity during HIV-1 infection (125) (408).

CMV in HIV-1 infected infants

The risk of CMV infection is increased in HIV-1 infected infants; the prevalence of congenital infection in HIV-1 infected infants may be as high as 20% (149). This may be the result of increased CMV reactivation in HIV-1 infected mothers, or as a result of HIV-1 induced immunosuppression in the infant. CMV infection during primary infection with HIV-1 in neonates is associated with an accelerated course of HIV-1 disease, and increased risk of mortality (93, 284, 293). CMV may contribute to HIV-1 disease progression in direct and indirect ways. CMV may broadly activate the immune system, providing a larger pool of activated cells that could be targets for

HIV-1 infection. CMV may also directly act as a co-factor for HIV-1 at the molecular or cellular level by activating latent provirus or altering HIV-1 tropism(218). The US28 gene of CMV encodes a protein homologous to CCR5 and may provide an additional co-receptor for HIV-1 infection. Though this has been demonstrated *in vitro* there is no evidence showing this occurs *in vivo*.

In adults, CMV viral load strongly predicts disease progression and mortality, and the effect was shown to be independent of HIV-1 viral load (159, 512). The relationship between CMV viral load and survival has not been evaluated in HIV-1 infected infants. The mechanism by which CMV may modify HIV-1 disease progression is unknown.

STUDY QUESTIONS AND SPECIFIC AIMS:

The natural history of infant HIV-1 infection

Paediatric HIV-1 infection causes rapid disease progression in the absence of antiretroviral (ARV) therapy. African cohorts have reported the highest rates of mortality, from 20-50% at 2 years of life. Understanding the pathogenesis in HIV-1 infected children is important to the design of prevention, treatment, and vaccination strategies. A cohort of 476 Kenyan children born to HIV-1 infected women was studied longitudinally from the time of birth to 24 months.

Specific aims for Chapter 2:

- Determine the prevalence and timing of HIV-1 transmission in a cohort of breastfed infants born to women receiving short course antenatal AZT.
- Describe the natural history of HIV-1 infection in a cohort of Kenyan infants infected through mother to child transmission by sequential viral load measurements and CD4 percentages.
- Determine correlates of infant mortality in the cohort.

CMV infection in a cohort of HIV-1 exposed Kenyan infants

The acquisition of CMV is common during infancy in sub-Saharan Africa. In regions of high HIV-1 prevalence, infants are at a high risk for co-infection with both pathogens. HIV-1 infected infants co-infected with CMV have an increased risk of mortality, though the mechanism governing this observation is unknown. CMV viral

load predicts death in adults co-infected with HIV-1. This relationship has not been determined in infants.

Specific aims for Chapter 3:

- Determine the prevalence of CMV infection in Kenyan infants born to HIV-1 infected women.
- Determine if HIV-1 infection influences the timing of CMV acquisition.
- Compare CMV viral loads longitudinally over time in infants with HIV-1 infection and uninfected infants.
- Examine the correlation between HIV-1 and CMV viral load.
- Determine if timing of CMV acquisition, or the magnitude of CMV viral load predict mortality in HIV-1 infected infants.

Lymphocyte distributions in healthy and HIV-1 exposed Kenyan infants

T cell phenotype can be described by the detection of cellular proteins using flow cytometry. Lymphocyte distributions change in response to age, environmental factors, and infection. The establishment of age-normal values for T cell phenotypes is important in understanding the effects of viral infection. The study of cellular phenotype can enable a better understanding of viral pathogenesis in infants.

Specific aims for Chapter 4:

- Using multi-colour flow cytometry, describe the phenotype of CD4 and CD8 T cells in healthy HIV-1 negative and HIV-1 exposed uninfected Kenyan infants.

- Compare phenotypic frequencies before and after detection of CMV.
- Examine the correlation between CMV viral load and frequencies of activated cells during acute and chronic CMV infection.

HIV-1 induced lymphocyte redistribution in Kenyan infants

The study of HIV-1 induced changes in lymphocyte subsets is valuable to the understanding of HIV-1 pathogenesis in infants.

Specific aims for Chapter 5:

- Using multicolour flow cytometry, compare phenotypic distributions of CD4 and CD8 T cell subsets between HIV-1 infected and exposed uninfected Kenyan infants during the first year of life.
- Examine the correlation between CMV viraemia and cellular activation during acute and post-acute HIV-1 infection.

HIV-1 specific CD8 T cell responses in Kenyan infants

The rapid course of HIV-1 infection in infants suggests HIV-1 specific CD8 responses may be less efficient in containing viraemia compared to adults. The deficiency in CD8 responses could be due to low frequencies of HIV-1 specific effector cells or an altered phenotype.

Specific aims for Chapter 6:

- Using class I HLA-tetramers, quantify HIV-1 specific CD8 T cells in HIV-1 infected infants during the first year of life.
- Compare frequencies of tetramer + cells with results from IFN- γ ELISpot assays in the children.
- Using multicolour flow cytometry describe the phenotype of HIV-1 specific CD8 T cells during the course of infection.

Immunophenotyping using multicolour flow cytometry

Multicolour flow cytometry is a powerful method for describing cellular phenotype. Successful use of this method requires careful optimisation of techniques and validation of assays. The large volumes of data generated using this method require well-planned data management systems.

Specific aims for Chapter 7:

- Describe the generation of the novel class I HLA-A*2902 tetramer.
- Describe optimisation and validation experiments used to design the phenotypic assays.
- Describe the integrated data systems generated for the collection, storage, management and retrieval of flow cytometry, clinical and other laboratory data.

Chapter 2

The Nairobi Mother-Infant Cohort: The Natural History of Infant HIV-1 Infection

Introduction

The Nairobi mother-infant cohort (CTL cohort) was established in 1999 by the Universities of Nairobi, Washington and Oxford. At the time of its inception, HIV-1 prevalence in Kenya had peaked at 15% (548) and the Government of Kenya (GoK) was not yet able to provide antiretrovirals (ARVs). Rates of mother-to-child transmission of HIV-1 (MTCT) were very high; a landmark study performed in the same clinics had recently revealed the cumulative risk of HIV-1 transmission at 24 months in breastfed infants to be 36.7% (390). Follow-up studies were aimed at identifying correlates of MTCT. Studies from commercial sex workers and discordant couples suggested virus specific T cell responses may provide protection from HIV-1 infection in highly exposed individuals (42, 183, 208, 276, 456). It was proposed that these responses could be protective in other highly exposed groups such as breastfed infants (299, 300, 457). The CTL cohort was thus established with 2 major goals: 1) to identify immune factors providing protection from HIV-1 acquisition (163-165), and 2) to identify correlates of disease progression(333, 401).

The intensive clinical follow up and specimen collection in this cohort enabled the construction of a model for HIV-1 infection in ARV-naïve breastfed infants. This chapter describes the natural history of HIV-1 infection in infants from the CTL cohort during the first two years of life. This chapter will not provide a comprehensive review of the cohort, previously published data is referenced, and data

presented here will be limited to those facets relevant to the aims of this thesis, specifically: 1) rates and timing of HIV-1 transmission 2) HIV-1 viral load and CD4 values and 3) mortality in the cohort. These three sections form the setting against which this thesis aims to understand infant immune development in the setting of HIV-1 and how the infant immune system responds to HIV-1 viral replication.

Materials & Methods

Enrolment and follow up

The ethics review committees of the Universities of Washington and Nairobi approved the use of human subjects for this study. Women attending Nairobi City Council clinics for antenatal care were offered HIV-1 counseling and testing, women screened before 28 weeks gestation who were HIV-1 seropositive and planned to live in Nairobi for two years after delivery were invited to enroll in the study (407). Counseling was provided to support safe infant feeding. Azidothymidine (AZT) was provided during the final trimester to reduce the risk of HIV-1 transmission (492). Women delivered their infants at Kenyatta National Hospital (KNH) under the care of study-trained midwives and neonatal examinations were performed by study paediatricians. Women and children visited the study clinic monthly for postnatal care and follow up. During each visit mothers provided information regarding infant feeding and reported illnesses. Postnatal care for mothers and infants included blood counts, RNA viral loads, routine infant vaccinations (418), and the provision of trimethoprim sulphamethoxazole (TMP-SMX) prophylaxis for the prevention of *Pneumocystis jiroveci* pneumonia (PCP) (88). All patients were followed for 1 year after delivery and blood specimens were obtained at birth (cord and venous), months

1, 3, 6, 9, and 12. HIV-1 infected infants and their mothers were followed up for an additional year at 3-monthly intervals.

In the case of a maternal or infant mortality, clinic and hospital records were reviewed to determine a likely cause of death. If a child died outside of hospital, cause of death was evaluated by verbal autopsy interviews with the next of kin. The limitations of verbal autopsies in delineating specific causes of death have been described (508) but difficulties in obtaining consent for postmortems necessitated this method.

HIV-1 diagnosis and quantification

During enrolment between 1999 and 2002, the laboratory facilities in Nairobi were unable to provide adequate diagnostic services for infant HIV-1. Dr. Julie Overbaugh's laboratory at the Fred Hutchinson Cancer Research Center (FHCRC, Seattle, Washington) provided HIV-1 diagnostics and RNA measurements. Infant HIV-1 infection was diagnosed by nested PCR amplifying HIV-1 gag DNA from dried blood spotted on filter paper (131). HIV-1 RNA viral loads were measured in cryopreserved plasma specimens, using the Gen-Probe assay (157). This assay has a limit of detection of 50 copies/ml. HIV-1 infection in infants was diagnosed as the detection of either HIV-1 DNA or RNA, followed by a positive specimen at the subsequent visit. *In utero* transmission was defined by the detection of HIV-1 DNA or RNA in the sample obtained on the day of birth. Infants testing negative at birth and positive at 1 month probably represent a mixed group, comprised of late *in utero*, *intrapartum*, and early breast-milk transmissions.

Blood counts

Blood counts were performed by the WHO Collaborative Centre Research and Training Laboratory in the Department of Medical Microbiology of the University of Nairobi. CD4+ and CD8+ T cell counts and percentages were determined at each visit following HIV-1 diagnosis using TriTest CD3FITC/CD8PE/CD45PerCP antibodies (BD Biosciences, San Jose, California, USA) and FACScan analysis with CELLQuest Software (BD Biosciences).

Data management and analysis

Clinical and laboratory data were collected and stored in a Microsoft Access Database (Microsoft Inc., Redmond, Washington, USA) and analysed using Stata SE v.9 (Stata Corp. College Station, Texas, USA). HIV-1 RNA viral loads were log₁₀-transformed for figures and comparison tests. Proportions were compared using Fisher's exact test, continuous variables were compared using Mann-Whitney U test. Kaplan-Meier survival analyses and Cox regression were used to describe time to infection, viral load peak and set-point, and death. Outcome data was censored if a child exited the study before 24 months and there was no record of death. Lowess curves fitted with running means were used to describe viral load and CD4 trends over time.

Results

Enrolment and follow-up

The CTL cohort was recruited from Nairobi City Council clinics between 1999 and 2002. During this time 31,731 pregnant women were screened for HIV-1 infection. Seroprevalence in the Nairobi antenatal clinics was 14%. A total of 510 women were enrolled in the study, 476 live singleton births were delivered, and 465 were observed for at least 1 year post partum.

Rates and timing of HIV-1 transmission

There were 90 HIV-1 transmission events recorded during study follow-up (19.4%). Timing of infection was available for 87 infants, for subsequent analyses children were categorised by: *in utero* (positive at birth = 30) *peri partum* (negative at birth positive at 1 month = 41) and late (first positive after 1 month = 16) infection.

Survival analysis was performed to describe the effect of breastfeeding on timing of HIV-1 infection (Figure 2.1) Timing of HIV-1 infection was estimated by taking the midpoint between the age at the last negative diagnosis and the first positive diagnosis. The majority of transmissions occurred by 1 month of age, with a median time to first HIV-1 infection of 0.49 months (95% CI = 0.46-0.52). Data for feeding status was available on 86 of the infants. No significant difference was detected for timing of HIV-1 infection in the formula (13 infants) vs breastfed (73 infants) groups (breast group median = 0.49 months CI = 0.47-0.52, formula group median = 0.46 months CI = 0.16-0.76, $p = 0.52$).

Viral loads during the first two years of life

HIV-1 RNA viral loads were retrospectively measured in all cryopreserved plasma specimens. Overall, there were 384 measurements of infant viral load in the HIV-1 infected infants during the study period, with a median of 4 observations per infant (range 1-9). Viral load during early infection strongly predicts time to AIDS and survival in both infants and adults (410, 443, 473, 496). Two variables were created to summarise the containment of viral load during early infection, “peak” (to summarise the viral load burst size) and “set-point” (as a surrogate for steady state viral load). Peak viral load was defined as the maximum viral load measured within 6 months of infection. As an estimation of an infant’s steady state viral load, set-point was defined as the first viral load observation measured at least 6 weeks after the peak. The pattern of viral load in the cohort differed remarkably from that observed in adults undergoing primary HIV-1 infection. There was great heterogeneity between patients with some infants having a net reduction in viral load, and others a net increase during follow up. Hence “set-point” used here is not completely

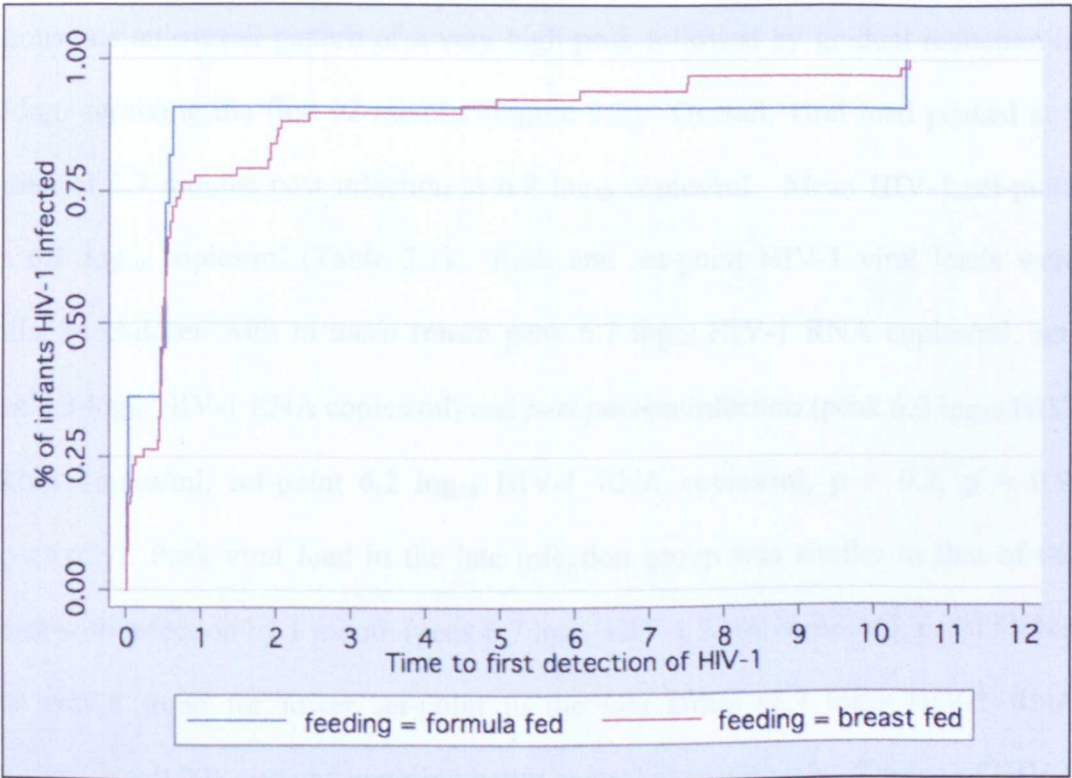


Figure 2.1. Timing of HIV-1 infection in the CTL cohort.

KM analysis is used to estimate timing of HIV-1 infection in 87 infants. Infants were grouped by mode of feeding, log rank $p = 0.52$ for comparison of breastfed (red line) vs non-breastfed (blue line).

Group	Peak			Set-point			Time to set-point	
	Mean \pm SE	Range	Median (IQR)	Mean \pm SE	Range	Median (IQR)	Mean \pm SE	Range
All HIV+	4.4 \pm 0.079	3.0-5.2	4.7 (3.5-5.0)	6.1 \pm 0.094	5.4-6.8	6.4 (5.9-6.9)	3.4	3.0-4.2
In utero	3.7 \pm 0.15	3.0-4.4	3.9 (3.4-4.4)	5.3 \pm 0.13	5.0-5.8	5.6 (5.1-6.1)	6.2	6.0-6.8
Post-partum	4.9 \pm 0.10	4.2-5.1	4.8 (4.3-5.3)	6.2 \pm 0.13	5.8-6.8	6.8 (6.3-7.3)	3.0	2.5-3.4
Late	6.7 \pm 0.19	5.8-7.5	7.0 (6.5-7.5)	7.7 \pm 0.11	7.4-8.2	8.0 (7.5-8.5)	4.5	4.2-5.1

Table 2.1. Summary of HIV-1 viral load indicators in HIV-1 infected infants.

All values are given in months. * $p < 0.0001$, ** $p < 0.0001$, In utero vs late $p < 0.04$, In utero vs post-partum $p < 0.0001$.

analogous to that defined elsewhere in adults. HIV-1 viral loads were persistently high during primary infection in the infants; longitudinal models fitted to data demonstrate an overall pattern of a very high peak followed by gradual reduction in children surviving the first 12 months (Figure 2.2). Overall, viral load peaked at a median of 1.7 months post infection at 6.8 log₁₀ copies/ml. Mean HIV-1 set-point was 6.1 log₁₀ copies/ml (Table 2.1). Peak and set-point HIV-1 viral loads were similar in children with *in utero* (mean peak 6.7 log₁₀ HIV-1 RNA copies/ml, set-point 6.3 log₁₀ HIV-1 RNA copies/ml) and *peri partum* infection (peak 6.9 log₁₀ HIV-1 RNA copies/ml, set-point 6.2 log₁₀ HIV-1 RNA copies/ml, *p* = 0.2, *p* = 0.9, respectively). Peak viral load in the late infection group was similar to that of the infants with infection by 1 month (peak 6.7 log₁₀ HIV-1 RNA copies/ml, *p* = 0.5), but there was a trend for lower set-point in the late group (5.7 log₁₀ HIV-1 RNA copies/ml, *p* = 0.09), perhaps implying better control over viraemia. Timing of HIV-1 acquisition affected time to peak and set-point viral load. HIV-1 viral load peaked later in the *in utero* group (3 months) compared to those with *peri partum* (0.58 months, *p* = 0.0001) or late (1.6 months, *p* = 0.04) infection. Children with HIV-1 *in utero* also had longer time to set-point compared to those with *peri partum* infection (6.2 vs 3.0 months, *p* < 0.0001).

Group	Peak		Time to peak ^a		Set-point		Time to set-point ^b	
	Mean ± SE	Range	Median	IQR	Mean ± SE	Range	Median	IQR
All HIV+	6.8 ± 0.079	5.0-8.4	1.7	0.58-3.0	6.1 ± 0.094	3.4-8.0	5.4	2.6-6.2
<i>In utero</i>	6.7 ± 0.15	5.0-8.4	3.0	1.3-6.0	6.3 ± 0.13	5.0-8.0	6.2	6.0-9.0
<i>Peri partum</i>	6.9 ± 0.10	5.2-8.1	0.58	0.47-2.6	6.2 ± 0.13	5.8-8.0	3.0	2.5-5.4
Late	6.7 ± 0.19	5.8- 8.0	1.6	1.0-3.0	5.7 ± 0.31	3.4-6.9	4.8	4.2-6.3

Table 2.1. Summary of HIV-1 viral load indicators in HIV-1 infected infants.

All values are given in months. ^a*in utero* vs *peri partum* *p* = 0.0001, *in utero* vs late *p* = 0.04, ^b*in utero* vs *peri partum* *p* < 0.0001.

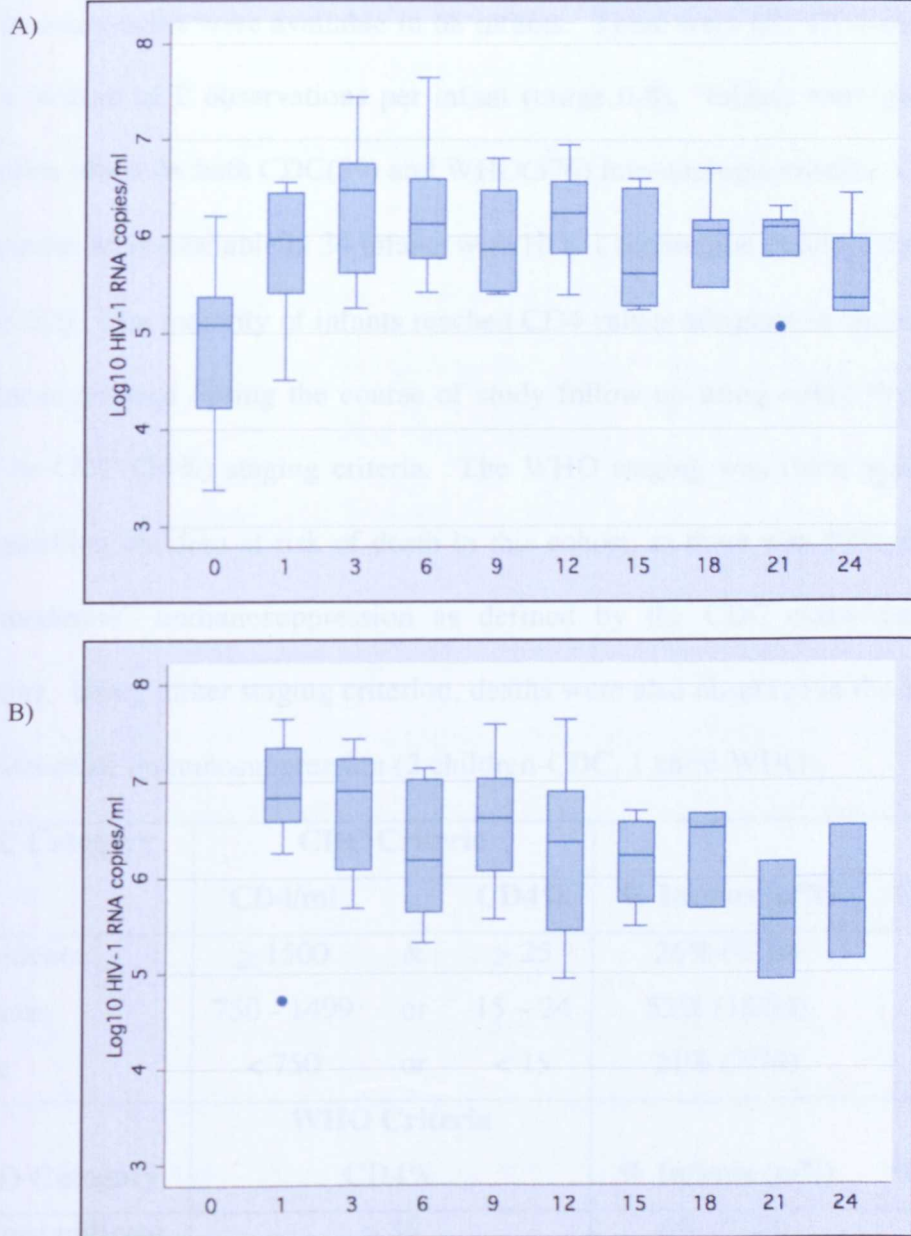


Figure 2.2. Plasma HIV-1 RNA viral loads during the first 2 years of life.

Data are shown for infants surviving 12 months of observation. Box plots show median line, upper and lower box hinges stretch to 25th and 75th percentiles, whiskers show upper and lower adjacent values, outliers are shown as closed circles. A) *in utero* infection group (17 children) B) peripartum infection group (26 children).

CD4 and immunosuppression

CD4 measurements were available in 68 infants. There were 685 CD4 observations, with a median of 2 observations per infant (range 0-8). Infants were grouped into categories based on both CDC(89) and WHO(576) immunologic criteria. CD4 values at 6 months were available in 34 infants with HIV-1 acquisition before 1 month of age (Table 2.2). The majority of infants reached CD4 values adequate to classify them as immunosuppressed during the course of study follow-up using either WHO (32/34, 94%) or CDC (74%) staging criteria. The WHO staging was more appropriate in distinguishing children at risk of death in this cohort, as there was 50% mortality in the “moderate” immunosuppression as defined by the CDC classification (9/18 children). Using either staging criterion, deaths were also observed in the group with no evidence of immunosuppression (2 children-CDC, 1 child-WHO).

CDC Category	CDC Criteria			% Infants (n/N)	% Deaths (n/N)
	CD4/ml		CD4%		
No evidence	≥ 1500	&	≥ 25	26% (9/34)	22% (2/9)
Moderate	750 - 1499	or	15 – 24	53% (18/34)	50% (9/18)
Severe	< 750	or	< 15	21% (7/34)	71% (5/7)
WHO Category	WHO Criteria			% Infants (n/N)	% Deaths (n/N)
	CD4%				
None/insignificant	> 35			6% (2/34)	50% (1/2)
Mild	30 – 35			3% (1/34)	0%
Advanced	25 – 29			26% (9/34)	11% (1/9)
Severe	< 25			65% (22/34)	64% (14/22)

Table 2.2. Immunologic indicators of immunosuppression in the cohort.

Infants with HIV-1 detection by 1 month were categorised using CD4% and CD4 cells/ml data at 6 months of age based on the 1994 CDC(89) and 2006 WHO(576) staging criteria.

Infant mortality

Despite frequent clinical follow-up and TMP-SMX prophylaxis, mortality was very high in the cohort; 33 (38%) died during the first year of life, cumulative mortality at 2 years was 54% (47 infants). Infant mortality was highest among the children with *in utero* HIV-1 infection (20/30, 67%), followed by the *peri partum* (23/41, 56%) and late (4/16, 25%) infection groups. Mortality in the late group was significantly lower than the *in utero* ($p = 0.01$) or *peri partum* ($p = 0.04$). Overall, infants with infection by one month of age had a greater risk of death at 2 years (61%, 43/71 infants) than those with late infection (25%, 4/16, OR = 4.6 CI = 1.4-16).

A description of the maternal and infant correlates of infant mortality in this cohort have been published (401). This paper also describes morbidity and causes of death in the cohort and will not be revisited here in detail. The predictors of infant mortality emerging in the multivariate model were maternal CD4 count < 200 cells/ml (HR = 2.7, $p = 0.03$) and delivery complications (HR = 3.4, $p = 0.005$). Infant CD4 percentage $\leq 15\%$ at 6 months was significant in the univariate model (HR = 5.5, $p = 0.01$). A decline in CD4 percentage of more than 10% was associated with a 3-fold greater risk of mortality (HR = 3.0, CI= 1.2-7.1).

The above report was published before plasma viral loads had been measured, infant viral load was thus not included in these models. The impact of viral load on infant mortality is examined in this section, in combination with CD4 values. In agreement with the published report, data here are limited to infants who survived at least 6 months of follow up to provide sufficient follow up data for group comparisons (68 infants).

Survivors had better control of HIV-1 RNA viral load and less precipitous CD4 declines than non-survivors (Figure 2.3). Mean peak and set-point HIV-1 viral loads were higher in the infants who died during follow up (Table 2.3). The mean peak viral load in the mortality group was 7.0 log₁₀ HIV-1 RNA copies/ml and 6.6 log₁₀ HIV-1 RNA copies/ml in the survivors (p = 0.01). The mean set-point viral load was 6.4 log₁₀ HIV-1 RNA copies/ml in the mortality group and 5.9 log₁₀ HIV-1 RNA copies/ml in the survivor group (p = 0.002). Time to peak and set-point were similar between survivors and mortalities (p > 0.05 for each). Median CD4 percent (13% vs 25%) and absolute count (842 vs 1569 cells/ml) at 6 months were lower in the infants who died.

	Mortalities	Survivors	
	Mean or Median	Mean or median	Comparison (p)
	(±SE) or (IQR)	(±SE or IQR)	
Peak HIV-1 viral load	7.0 ± 0.12	6.6 ± 0.11	0.01
Set-point HIV-1 viral load	6.4 ± 0.14	5.9 ± 0.13	0.002
Time to peak	3.0 (0.74-5.6)	2.4 (0.80-3.0)	0.2
Time to set-point	5.5 (2.7-8.7)	5.6 (2.6-6.2)	> 0.9
CD4% at 6 months	13 (9.0-21)	25 (19-28)	0.05
CD4 cells/ml at 6months	842 (623-1440)	1569 (1170-2200)	0.007

Table 2.3. Indicators of HIV-1 disease progression in infants dying before, or surviving 2 years of life.

Cox regression was used to explore factors influencing infant survival time. The subset of 65 infants provided 1092 person-months of observation, with an overall incidence rate of 26.5 deaths/1000 person-months. Although there were more deaths in the group with HIV-1 acquisition before 1 month, mean survival time in the three groups was similar: *in utero* group 19 months (16-21), *peri partum* group 23 months

(14-20), low group 21 months (12-34), $p = 0.001$ (Table 2). *Survival* (Table 2). As mentioned above, CD4 values at 6 months were a strong predictor of

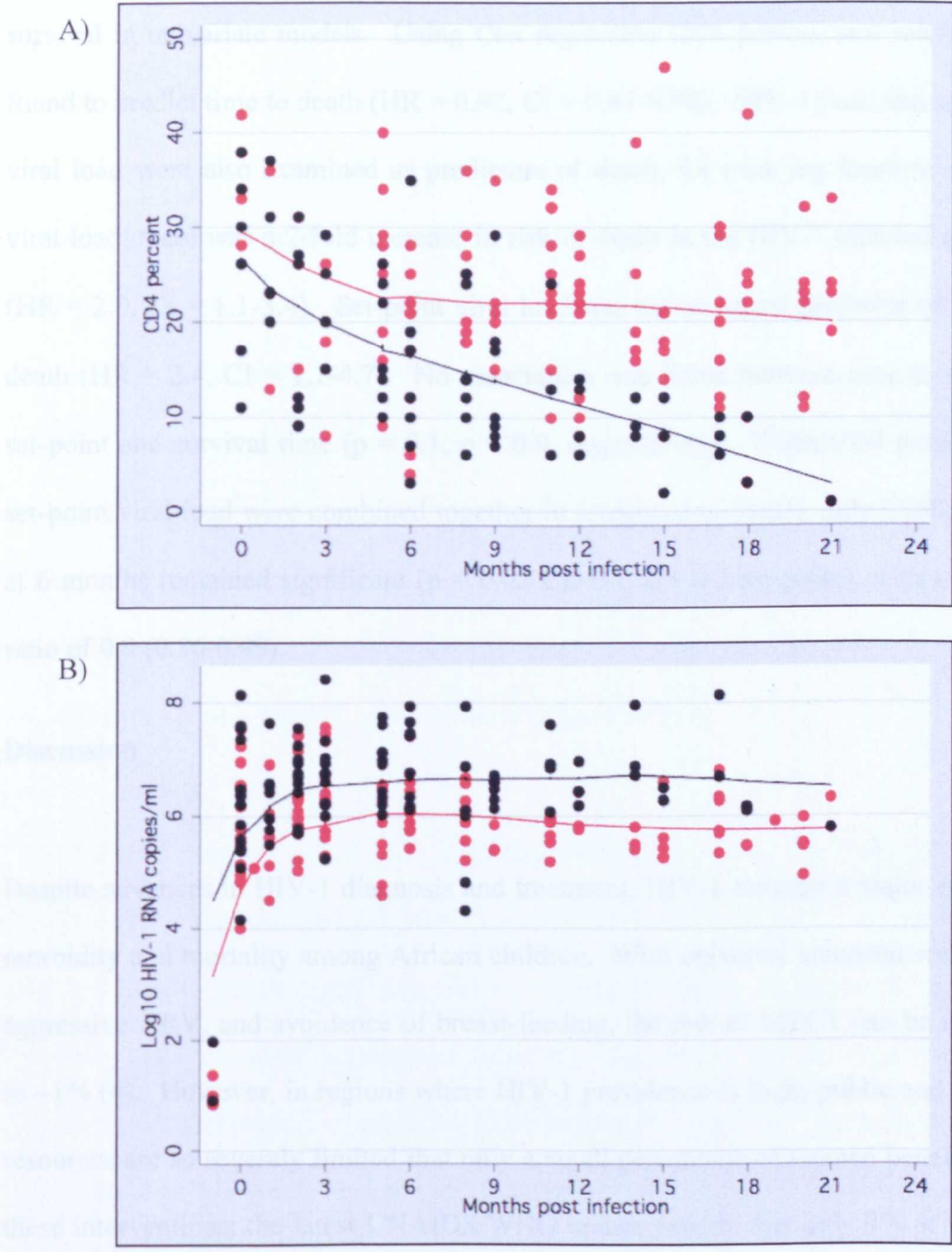


Figure 2.3. CD4 values and viral loads in children surviving or dying during the first two years of life.

Scatter plots with lowess curves fitted to mean A) CD4 percent and B) HIV-1 RNA viral load from time of first detection to 24 months. Red = survivors to 24 months, black = deaths after 6 months and before 24 months. Group shown is limited to children with first HIV-1 detection by 1 month of age.

(14-20), late group 21 months (17-24), ($p > 0.05$ for comparisons between each group). As mentioned above, CD4 percent at 6 months was a strong predictor of survival in univariate models. Using Cox regression CD4 percent at 6 months was found to predict time to death (HR = 0.92, CI = 0.87-0.98). HIV-1 peak and set-point viral load were also examined as predictors of death, for each log increase in peak viral load, there was a 2-fold increase in risk of death in the HIV-1 infected children (HR = 2.0, CI = 1.1-3.4). Set-point viral load was the strongest predictor of time to death (HR = 2.4, CI = 1.1-4.7). No association was found between time to peak or set-point and survival time ($p = 0.1$, $p = 0.9$, respectively). When CD4 percent and set-point viral load were combined together in a regression model, only CD4 percent at 6 months remained significant ($p = 0.02$ CD4%, $p = 0.2$ set-point), with a hazard ratio of 0.9 (0.86-0.99).

Discussion

Despite advances in HIV-1 diagnosis and treatment, HIV-1 remains a major cause of morbidity and mortality among African children. With universal antenatal screening, aggressive ARV, and avoidance of breast-feeding, the risk of MTCT can be reduced to ~1% (4). However, in regions where HIV-1 prevalence is high, public and private resources are so severely limited that only a small percentage of women benefit from these interventions; the latest UNAIDS/WHO update reports that only 9% of women globally are able to access these interventions(549). Consequently, the majority of pregnant women deliver without any HIV-1 support, and the number of infected children is growing. Paediatric HIV-1 prevention, treatment, and vaccination thus remain a global health priority. An understanding of natural infection is invaluable to

both treatment and vaccination strategies. This chapter describes the natural history of HIV-1 infection in ARV-naïve Kenyan children.

Though antenatal AZT was provided to the mothers, a number of children became infected during the study. At birth 6.5% of the children in the current study were found to be infected with HIV-1, which is in range of the figure reported from the Thai short-course AZT study (4.8% at birth(492)). Additional transmissions *intrapartum* and through breastfeeding resulted in an overall transmission frequency of 20%. There were very few transmissions after one month of age and the overall timing of transmission was similar between breast-fed and formula-fed children. This observation is consistent with other reports demonstrating that virus levels are highest in the colostrum and early milk(454) and the risk of transmission through breastfeeding after 1 month is lower but consistent(390).

The natural history of infant HIV-1 differs substantially from the typical course of HIV-1 observed in infected adults(410, 411, 453, 496) (Figure 2.4) Several factors may explain the more aggressive disease course observed in infants with HIV-1. Infection via the oral or gut mucosa *in utero*, *intrapartum*, or via breast milk may accelerate CD4 depletion of the GALT. Infection *in utero* may cause damage to T cell repertoires by death of infected thymocytes(482) or negative selection of antigen-specific T cells(505). Genetic similarity between mother and infant may result in the transmission of variants with pre-adapted fitness for the new host (214, 319, 502). HIV-1 specific responses generated by young infants are less frequent and weaker in magnitude than in adults, but become stronger over time(78, 467), suggesting a maturing of cellular immunity with age. Weak CD4 Th1 type responses in neonates,

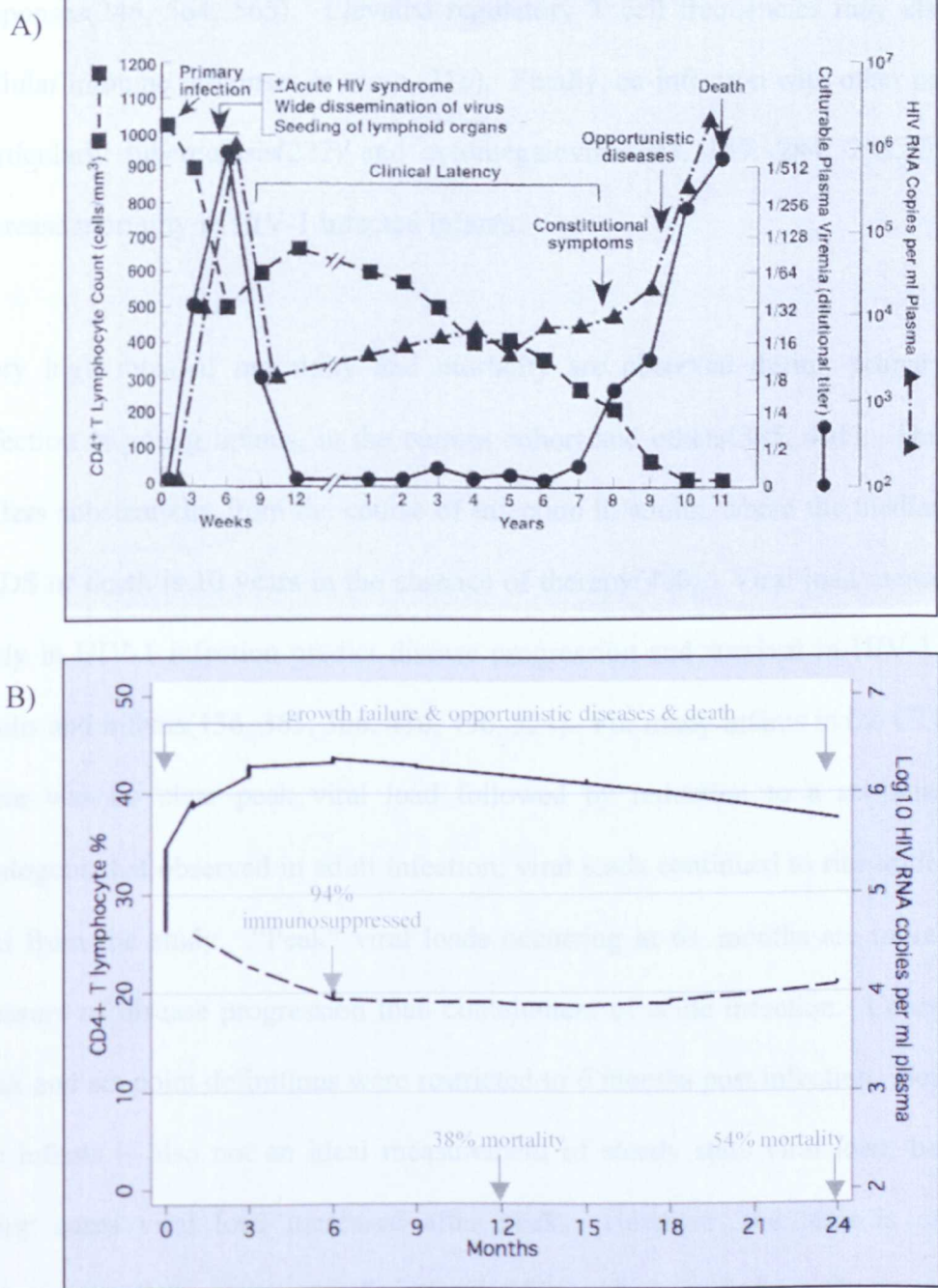


Figure 2.4. The natural history of HIV-1 infection in adults and infants.

Figures show HIV-1 viremia and CD4 values from HIV-1 infected adults and infants.

A) From Pantaleo, G., C. Graziosi, et al. (1993). "New concepts in the immunopathogenesis of human immunodeficiency virus infection." *N Engl J Med* 328(5): 327-335. B) from the current cohort, dashed line = CD4 percent, solid line = HIV-1 RNA viral load.

or responses skewed toward a Th2-type response may fail to support CD8 T cell responses(246, 564, 565). Elevated regulatory T cell frequencies may also inhibit cellular immune responses *in utero* (316). Finally, co-infection with other pathogens, particularly tuberculosis(222) and cytomegalovirus,(93, 149, 284, 293, 396) may increase mortality in HIV-1 infected infants.

Very high rates of morbidity and mortality are observed during primary HIV-1 infection in young infants, in the current cohort and others(395, 401). This pattern differs substantially from the course of infection in adults, where the median time to AIDS or death is 10 years in the absence of therapy(460). Viral load measurements early in HIV-1 infection predict disease progression and survival in HIV-1 infected adults and infants(136, 365, 366, 410, 496, 521). For many infants in the CTL cohort, there was no clear peak viral load followed by reduction to a set-point that is analogous that observed in adult infection; viral loads continued to rise until death or exit from the study. “Peak” viral loads occurring at 6+ months are more likely a measure of disease progression than containment of acute infection. Consequently, peak and set-point definitions were restricted to 6 months post infection. Set-point in the infants is also not an ideal measurement of steady state viral load, because in many cases viral load increased after peak. However, the term is useful for summarising the containment of viraemia achieved by some infants. Later acquisition of HIV-1 was associated with shorter time to peak and set-point viral loads, and this group had a greater drop from peak to set-point, implying better control over viraemia. The group with *in utero* HIV-1 acquisition had a longer time to peak and set-point viral load. This may be due to the suppressive effect of AZT acquired *in utero*. Peak and set-point viral load were strong predictors of time to death in the

cohort, each log₁₀ increase in either peak or set-point was associated with ~2-fold increased risk of death.

CD4 depletion is the hallmark of HIV-1 disease progression, and is highly predictive of mortality (162, 365, 431, 444). Though adult CD4 decline sharply during acute infection, their numbers are partially restored after set-point is reached, after which they slowly decline during the years of host-viral equilibrium preceding AIDS(167, 411). In the current cohort a consistent decline was observed in both CD4 percent and absolute counts/ml during study follow-up. By 6 months of age, 94% of infants met WHO definitions for immunosuppression, and CD4% at 6 months was predictive of mortality by 2 years of follow-up. Staging by WHO(576) and CDC(89) showed a very high risk for mortality in children with a broad range of CD4% frequencies. Opportunistic infections (OI) are uncommon at values above 500/ml in adults(378), but this does not appear to be the case in very young children who may develop OI and AIDS at relatively high CD4 values(382). Children living in resource-poor settings may be at an additional risk of co-infections due to increased exposure to bacterial pathogens, helminth infections, viral co-infections and tuberculosis.

At the time of this study enrolment and follow-up, ARVs were not widely available in Kenya. In 2004, ARVs became available to the GoK, which began a massive distribution programme through hospitals, research groups and NGOs. Women and children of the CTL cohort are now receiving ARVs at a number of treatment centres in Nairobi. Previous studies have demonstrated that mortality is reduced by the initiation of ARV early in life(169, 561), and the CDC recommends immediate treatment for all infants less than 12 months old(89), regardless of immunological

parameters of symptoms. Recent data from South Africa(558) demonstrate ARV initiated before 12 weeks of age can substantially reduce morbidity and mortality in young infants. The data presented here suggest that Kenyan infants are at an extremely high risk for death from HIV-1 during the first two years of life and must be included in national strategies for HIV/AIDS treatment.

Summary

This chapter describes viral load, CD4, and mortality trends in the CTL cohort. Early viral loads and CD4% at 6 months emerged as strong predictors of mortality. The following chapters explore potential factors that may contribute to the aggressive course of HIV-1 disease in very young children.

Chapter 3

Primary CMV infection:

A co-factor for HIV-1 disease progression in African infants?

Introduction

Cytomegalovirus (CMV) infection may be an important co-factor for disease progression in human immunodeficiency virus type 1 (HIV-1) infected children. Co-infection with CMV has been associated with a 3-fold increased risk of mortality during the first year of life(93, 149, 284, 293, 396). In adults, CMV viral load is predictive of HIV-1 disease progression and mortality (126, 159, 510). Though it is clear that CMV co-infection influences HIV-1 disease progression, the mechanism by which CMV influences HIV-1 progression in infants is unknown, and the quantitative relationship between levels of HIV-1 RNA and levels of CMV DNA in infants has not been determined.

If CMV presents a significant risk factor for HIV-1 disease progression, the impact might be greatest in African children where CMV is commonly acquired in infancy (40, 372). Infant mortality in HIV-1 infected infants in the absence of treatment is very high in sub-Saharan Africa (52% at 2 years(401)) compared to western cohorts (~15% at 18 months(249)) (395), and co-infections may contribute to excess mortality. The current study was performed to examine the impact of CMV co-infection and HIV-1 disease progression in Kenyan infants.

Materials & Methods

Participants

A subset of 44 HIV-1 infected (HIV+) and 20 HIV-1 exposed uninfected (HIVexp-) infants were selected from the CTL cohort who met the following criteria: survival to at least 3 months of follow up and a plasma specimen available within 1 month of birth. Specimens from 13 healthy HIV-1 uninfected Kenyan women were obtained from a separate cohort (MIP cohort) with similar characteristics(63).

HIV-1 diagnosis and quantification

Infant HIV-1 infection was diagnosed by nested PCR amplifying HIV-1 gag DNA from dried blood spotted on filter paper(131). HIV-1 RNA viral loads were measured in cryopreserved plasma specimens, using the Gen-Probe assay(157). HIV-1 infection in infants was defined as the detection of either HIV-1 DNA or RNA, followed by a positive specimen at the subsequent visit.

Infants were grouped according to first HIV-1 detection: *in utero* (sample collected within 48 hours of birth positive, 15 infants) *peri partum* (negative at birth, positive by 1 month, 16 infants) or late (after 1 month, 13 infants). Peak viral loads were defined as the highest viral load measured in the first 6 months of life. HIV-1 set-point was defined as the first viral load observation measured at least 6 weeks after the HIV-1 peak.

CMV diagnosis and quantification

The CMVScan (BD Diagnostics, Sparks, Maryland, USA) system, which detects CMV IgM & IgG antibodies, was used to estimate CMV seroprevalence in the HIV-1 infected and uninfected women. Thirteen HIV-1 uninfected women and 29 HIV-1 infected women were screened at delivery for the presence of CMV antibodies.

Nucleic acids were extracted from 200 µl of plasma using the Qiagen UltraSens virus extraction kit (Qiagen, Valencia, California, USA). CMV viral loads were obtained by quantitative PCR detecting the glycoprotein B gene (gB) as previously described(65). PCRs were performed in triplicate for each individual and the mean was calculated of the replicates. The lower limit of detection was 1 copy/reaction. Negative and indeterminate PCR assays were assigned a value equivalent to the midpoint between the limit of detection and zero. Peak CMV viral load was defined as the highest viral load observation for each infant.

Statistical analyses

SPSS v.11 (SPSS, Inc. Chicago, Illinois, USA) and Stata SE v.9 (Stata Corp. College Station, Texas, USA) for Macintosh were used for statistical analyses. Viral loads were base 10 log-transformed (Log_{10}) before comparisons. T-tests and Mann-Whitney U tests were used to compare continuous variables, Chi Square tests were used to compare proportions. Pearson correlation was used to measure independence between continuous variables. Kaplan-Meier and the log-rank test were used to compare time to CMV infection and death between dichotomized variables. Cox regression was

used to estimate the hazard rate of death. Median smoothing splines fitted with cubic curves were used to illustrate changes in viral load over time.

Results

Prevalence and transmission of CMV in the Nairobi mother-child cohort

Adult CMV seroprevalence in HIV-1 infected pregnant women was 97% (28/29) and 100% (13/13) in the uninfected women. CMV viral loads were measured at delivery in 13 HIV-1 uninfected and 64 HIV-1 infected women. None of the uninfected women from the control cohort had detectable CMV viraemia; 11/64 (17%) HIV-1 infected women had detectable plasma CMV viraemia. The mean CMV plasma viral load in the 11 viraemic women at delivery was 1.5 log₁₀ copies/ml (range 1.5-2.2).

Infants were serially monitored for CMV viraemia during the first two years of life. Despite an additional year of follow-up in the HIV+ group, the median number of observations in HIV+ and HIVexp- infant groups were similar due to the high rate of mortality in the HIV+ group (median of 6 observations/infant in each group). Overall, CMV viraemia was detected in the plasma of 95% (61/64) of the infants by 2 years of age (Table 3.1). The overall prevalence of CMV infection by 12 months of age was similarly high in both HIV+ (41/44, 93%) and HIVexp- (19/20, 95%) infants. CMV prevalence at 1 year was similar between infants acquiring HIV-1 *in utero* (15/15, 100%), *peri partum* (14/16, 88%), and late (12/13, 92%).

CMV was acquired early in life and most of the 42 co-infected infants acquired HIV-1 first (60%, 25/42 infants). Intrauterine acquisition of HIV-1 was associated with an increased detection of CMV viraemia at birth: 27% (4/15) with HIV-1 *in utero* vs

2.0% (1/49) of all other infants ($p = 0.009$). CMV and HIV-1 viraemia were detected concurrently in 9 infants (21%) and 8 infants (19%) acquired CMV first. CMV was detected earlier in infants with intrauterine acquisition of HIV-1 (median 0.5 months, 95% CI = 0.0, 1.3) compared to infants with *peri partum* (median 2.0, CI 1.7, 2.3, $p = 0.002$), late (median 2 months CI = 1.8, 2.2, $p = 0.02$), or no HIV-1 infection (median 2.0, CI 1.7, 2.3, $p = 0.001$).

CMV was detected in the blood of five infants at birth (4 HIV+, 1 HIVexp-). Detection of maternal plasma CMV viraemia at delivery was not associated with infant CMV detection at birth ($p = 0.6$). Four aviraemic mothers delivered infants with detectable CMV, 11 CMV viraemic mothers delivered infants without CMV detection, and only 1 mother-child pair was concurrently CMV viraemic at delivery.

Neonatal examinations were performed on all infants; no physical signs of congenital CMV were observed. The HIVexp- infant (B1-249) with CMV viraemia at birth survived and exited the study at 12 months, three of the four HIV+ infants died, one had failure to thrive and died with pneumonia at 15 months, one died with sepsis at 6 months, and one died with pneumonia at 7 months.

Cumulative percentage of infants with CMV viraemia (number)

	Uninfected	All HIV+	In utero	Peri partum	Late ^f	All infants
Birth	5% (1)	9.1% (4)	27% (4) ^d	0% (0)	0%	7.8% (5)
Month 1	20% (4)	30% (13)	53% (8)	13% (2)	23% (3)	27% (17)
Month 3^a	90% (18)	84% (37)	93% (14)	75% (12)	85% (11)	86% (55)
Month 6	90% (18)	89% (39)	93% (14)	81% (13)	92% (12)	89% (57)
Month 9	90% (18)	91% (40)	100% (15)	81% (13)	92% (12)	91% (58)
Month 12^b	95% (19)	93% (41)	100% (15)	88% (14)	92% (12)	94% (60)
Month 24^c	95% (19)	95% (42)	100% (15)	94% (15)	92% (12)	95% (61)
Total screened	20	44	15	16	13	64
Time to CMV viraemia^e	2.0 (1.7, 2.3)	2.0 (1.5, 4.1)	0.5 (0.0, 1.3)	2.0 (1.7, 2.3)	2.0 (1.8, 2.2)	2.0 (1.9, 2.1)

Table 3.1. Point-prevalence of CMV viraemia in the Nairobi mother-infant cohort during two years of follow up.

Cumulative percentage of infants CMV viraemic by study visit. Number of infants is shown in parentheses. ^aIncludes one infant with HIV-1 infection *in utero* who became CMV viraemic at 2 months. ^bIncludes one infant with HIV-1 infection at 1 month who became CMV viraemic at 10 months. ^cOne infant became CMV viraemic at 20 months. ^dInfants with HIV-1 detected at birth were more likely to be CMV viraemic at birth, $p = 0.009$. ^eMedian is shown with 95% CI. Log-rank test $p < 0.001$ for infants with intrauterine HIV-1 acquisition vs all others. ^fThe late infection group was comprised of infants infected between 2-11 months.

Impaired containment of CMV in HIV-1 co-infected infants

CMV viral loads were examined during the first two years of life. As a group HIVexp- were better able to control CMV replication than HIV+ infants (Figure 3.1). CMV viral loads rapidly decreased in the HIVexp- group during the first year of life while in the HIV+ group CMV viral loads declined more slowly. Peak CMV viral load was defined as the highest viral load measured for each infant during the first 6 months of infection (Figure 3.2). Overall, children with early HIV-1 infection (before 1 month) had significantly higher peak viral loads (3.1 log₁₀ copies/ml, IQR 2.6-3.5) than children with no HIV-1 infection (2.7 log₁₀ copies/ml, IQR 2.1-3.3, $p = 0.05$). Peak viral loads were similar in the late infection group (2.9 log₁₀ copies/ml, IQR 2.5, 3.1) and the HIVexp- children ($p = 0.5$). Median peak CMV viral load was 3.1 log₁₀ copies/ml (IQR 2.6-4.0) in the group with HIV-1 infection *in utero*, and 3.1 log₁₀ copies/ml (IQR 2.3-3.5) in the *peri partum* group.

Primary co-infection with HIV-1 and CMV

CMV infection was present in nearly all HIV+ infants. As discussed in Chapter 2, very high viral loads were maintained in the majority of infants with HIV-1 infection. In comparison, CMV viral loads were more efficiently controlled, with a steady decline to lower levels observed following acute infection (Figure 3.3). The extraordinary prevalence of CMV in the cohort, and the very early timing of transmission made comparisons of HIV-1 viral loads between CMV viraemic and aviraemic infants impractical, as only 2 HIV+ infants did not have detectable CMV viraemia. Pearson correlation was used to describe quantitatively the relationship

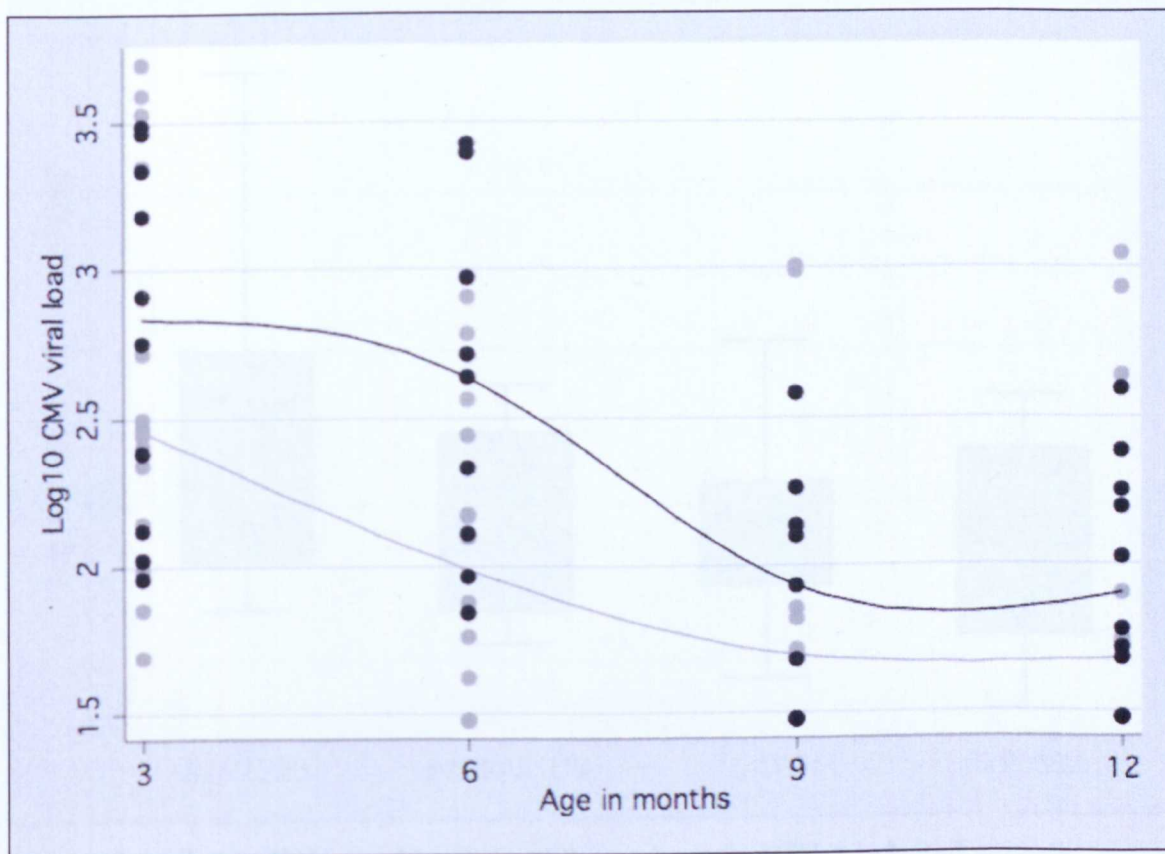


Figure 3.1. CMV viral loads during primary infection in HIV-1 infected and exposed uninfected infants.

CMV viral loads are shown for children with first detection of CMV viraemia at 3 months of age who survived 12 months of follow-up. Black line shows infants with HIV-1 acquisition before 1 month, gray line shows HIVexp- infants. Median smoothing splines are used to summarise data from the two groups of infants.

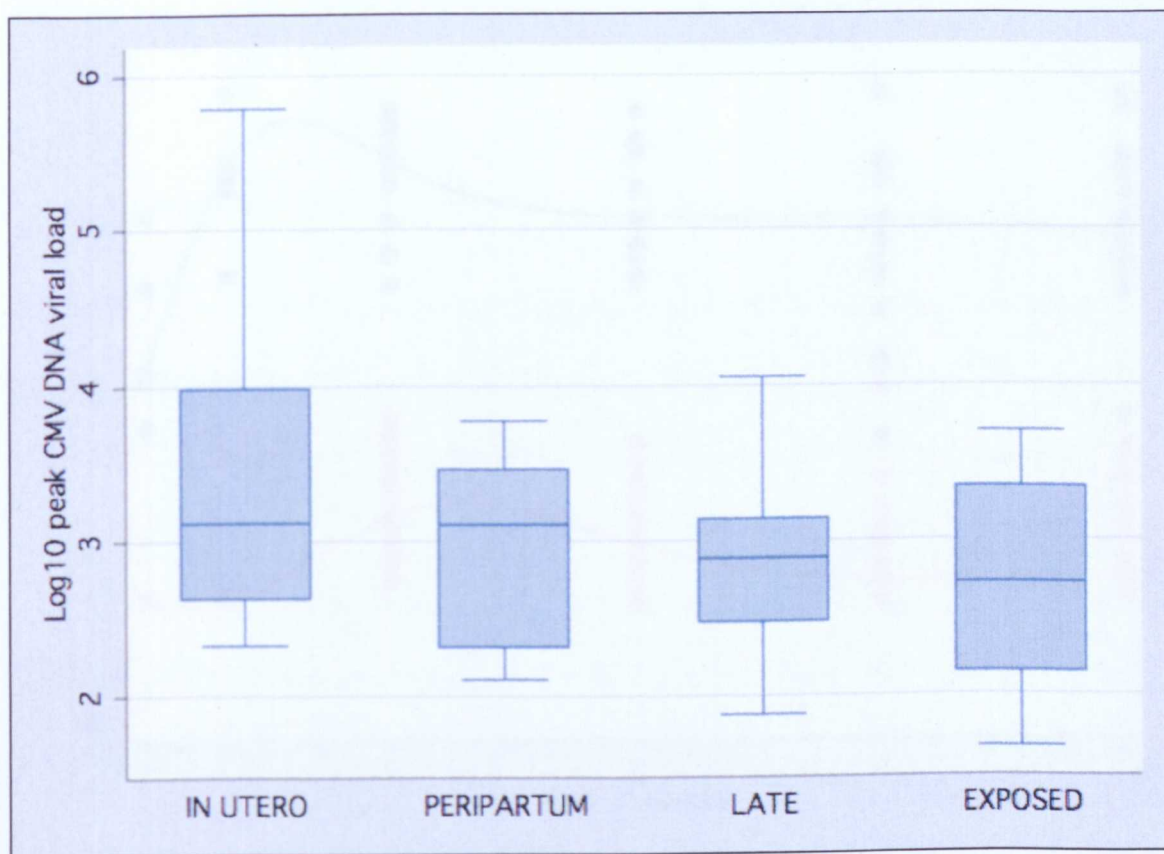


Figure 3.2. Peak CMV viral loads in children born to HIV-1 infected women.

Boxplots show median and 95% CI for infants grouped by HIV-1 exposure.

between CMV and HIV-1 viral load (Figure 3.4). Peak CMV viral load was positively correlated with HIV-1 peak ($p = 0.36$, $p = 0.02$) viral load, but not set-point

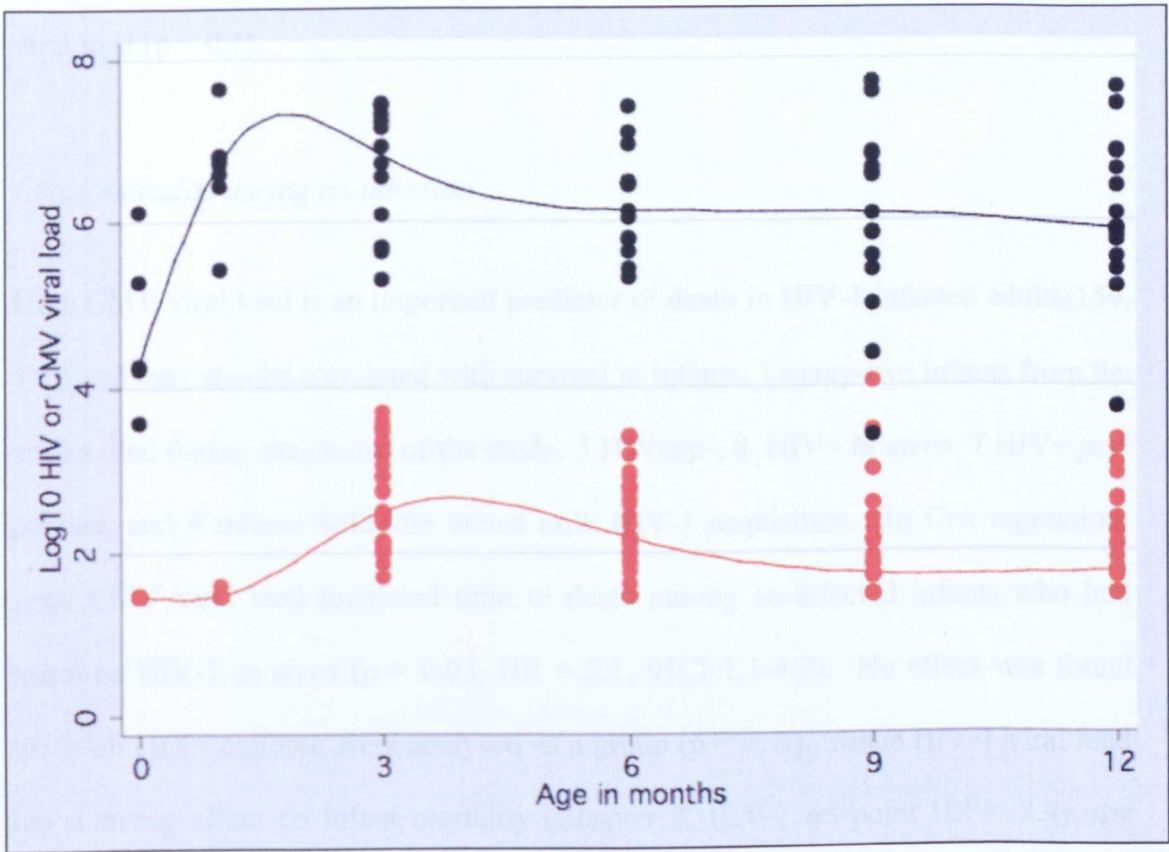


Figure 3.3. Longitudinal HIV-1 and CMV viral loads in co-infected children during the first two years of life.

HIV-1 (black) and CMV (orange) viral loads are shown with fitted median smoothing splines. Children shown here survived 12 months of follow-up and were diagnosed with HIV-1 infection by 1 month of age and CMV at 3 months.

The effect of timing of CMV acquisition was also examined. Only 4 children in the current study were infected with CMV at birth, so children were stratified by CMV diagnosis by 1 month of age or after (Figure 3.5). Without adjusting for HIV-1 viral load, there was a trend for increased mortality in children with early CMV acquisition among the group of all HIV-1 infected (OR = 1.5, 95% CI = 0.5-4.5). To account for HIV-1 viral load, infants were stratified based on HIV-1

between CMV and HIV-1 viral load (Figure 3.4). Peak CMV viral load was positively correlated with HIV-1 peak ($p = 0.36$, $p = 0.02$) viral load, but not set-point viral load ($p = 0.1$).

Infant mortality during co-infection

High CMV viral load is an important predictor of death in HIV-1 infected adults(159, 510) and may also be correlated with survival in infants. Twenty-two infants from the subset died during the course of the study: 3 HIVexp-, 8 HIV+ *in utero*, 7 HIV+ *peri partum*, and 4 infants with late breast milk HIV-1 acquisition. In Cox-regression, peak CMV viral load predicted time to death among co-infected infants who had acquired HIV-1 *in utero* ($p = 0.03$, HR = 2.1, 95CI 1.1-4.3). No effect was found when all HIV+ children were analysed as a group ($p = 0.3$). Since HIV-1 viral load has a strong effect on infant mortality (Chapter 2, HIV-1 set-point HR = 2.4), the survival analysis was adjusted for HIV-1 set-point viral load. All 42 children with CMV had a defined HIV-1 set-point viral load. The adjusted HR for peak CMV viral load was 1.7, and for HIV-1 set-point was 2.1, neither were significant ($p = 0.1$ CMV, $p = 0.074$ HIV-1), indicating effects of CMV viral load could not be discriminated from the strong effect of HIV-1 set-point in the current study (Figure 3.5).

The effect of timing of CMV acquisition was also examined. Only 4 children in the current study were infected with CMV at birth, so children were stratified by CMV diagnosis by 1 month of age or after Figure 3.5. Without adjusting for HIV-1 viral load, there was a trend for increased mortality in children with early CMV acquisition among the group of all HIV+ infants ($p = 0.06$) and those with HIV-1 before 1 month ($p = 0.07$). To control for HIV-1 viral load, infants were stratified based on HIV-1

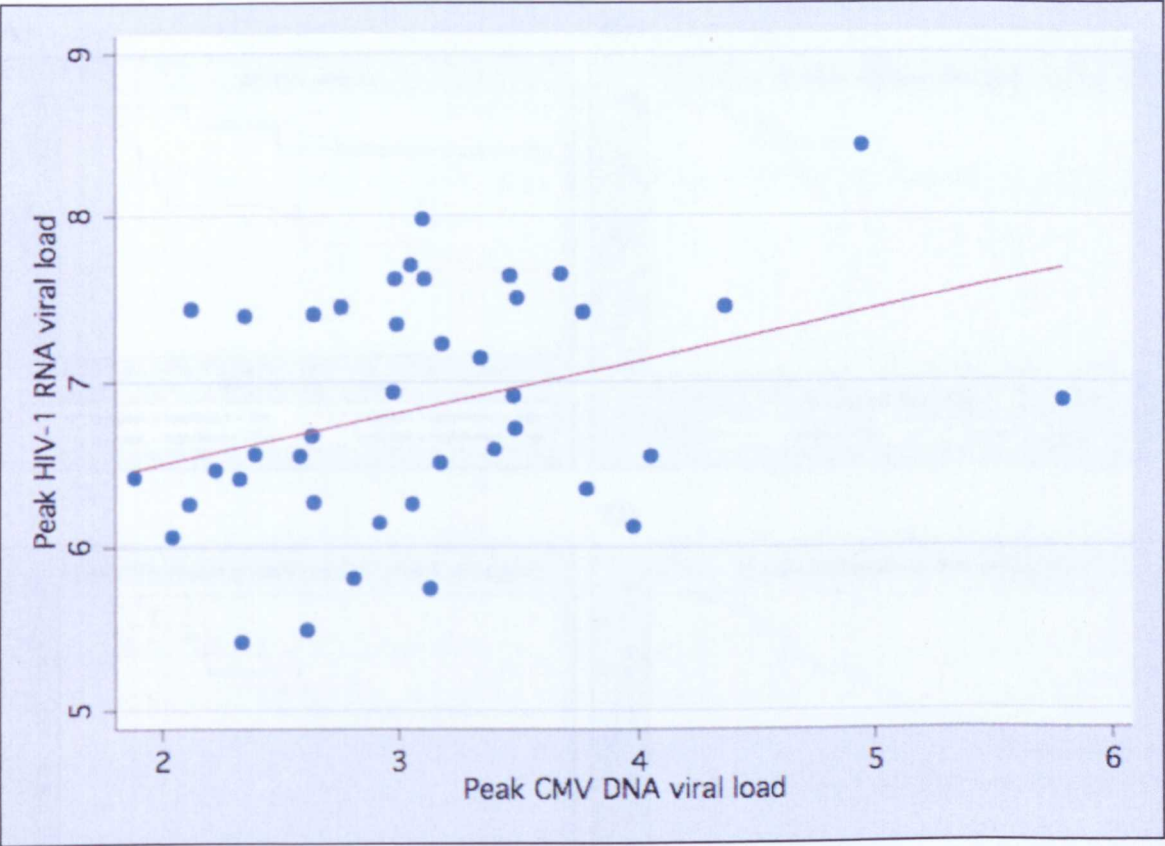


Figure 3.4. Correlation between CMV and HIV-1 viral load.

Scatter plot and linear prediction of HIV-1 and CMV viral loads in co-infected infants.
Spearman's $\rho = 0.36$, $p = 0.02$.

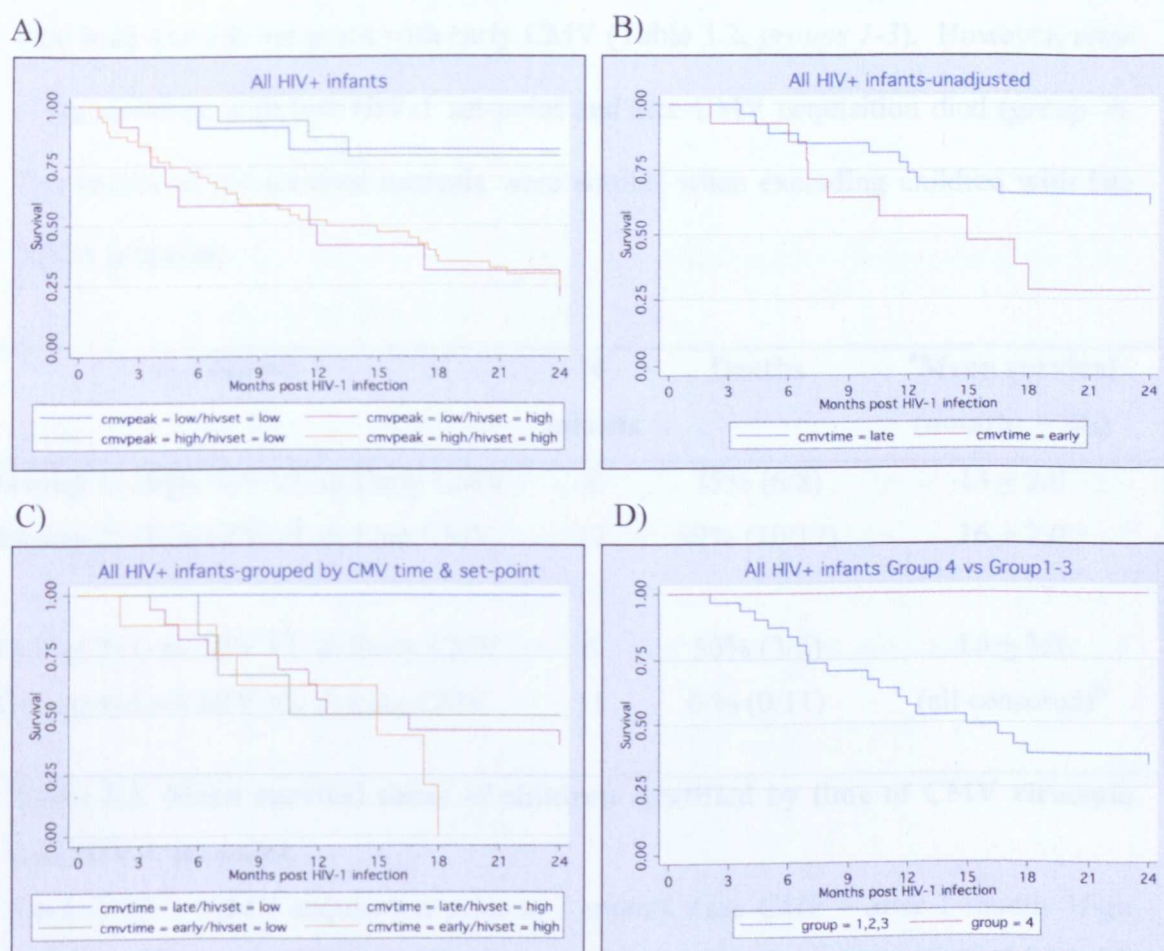


Figure 3.5. Predictors of mortality in co-infected infants.

A) Peak CMV-1 and set-point HIV-1 viral load were stratified above (*high*) or below (*low*) the group medians. B) Children stratified by timing of CMV acquisition before (*early*) and after (*late*) 1 month of age (log rank $p = 0.06$). C) Children stratified by timing of CMV acquisition and HIV-1 set-point viral load. D) Children grouped according to Table 3.2, log rank $p = 0.0013$ for comparison between group 4 and groups 1,2,3 combined. No deaths were observed in the children low HIV-1 set-point and late CMV acquisition.

set-point viral load above or below the cohort mean of 6.1 log₁₀ copies/ml (Table 3.2). There was no difference in survival between groups of infants with high set-point viral load and low set-point with early CMV (Table 3.2, *groups 1-3*). However, none of the children with low HIV-1 set-point and late CMV acquisition died (*group 4*). The results of the survival analysis were similar when excluding children with late HIV-1 infection.

Group	N infants	Deaths	^a Mean survival (months ± SE)
Group 1) High HIV VL & Early CMV	8	75% (6/8)	13 ± 2.0
Group 2) High HIV VL & Late CMV	17	59% (10/17)	16 ± 2.0
Group 3) Low HIV VL & Early CMV	6	50% (3/6)	16 ± 3.3
Group 4) Low HIV VL & Late CMV	11	0 % (0/11)	(all censored) ^b

Table 3.2. Mean survival times of children stratified by time of CMV viraemia and HIV-1 set-point.

Early CMV = CMV acquisition prior to 1 month, *Late CMV* = after 1 month. High and low HIV-1 set-point was defined as above or below the cohort mean of 6.1 log₁₀ copies/ml. ^aMean survival times are restricted to the follow-up of 24 months, children not dying before 24 months are censored, so true mean is underestimated. ^bNo children with low HIV-1 set-point and late CMV acquisition died, all data are censored so no mean survival time can be estimated.

Since children in groups 1-3 were similar in survival time and mortality, a Kaplan-Meier survival analysis was performed comparing group 4 to the combined groups 1-3. Having high viral load or early CMV acquisition greatly increased risk of death among all the children with HIV-1 ($p = 0.0013$), and those with early HIV-1 acquisition ($p = 0.0087$). It was not possible to calculate a hazard ratio because none of the infants in group 4 died (Table 3.3).

Time of HIV-1 infection	Comparison groups	N infants	^a mean survival (months \pm SE)	Log-rank p
All infections	Group 4 children vs	11	(all censored) ^b	p = 0.0013
	others	31	15 \pm 1.4	
Before 1 month	Group 4 children vs	7	(all censored) ^b	p = 0.0087
	others	23	15 \pm 1.6	

Table 3.3. Children with low HIV-1 set-point and late CMV acquisition have the best prognosis during the first two years of life.

Group 4 = children with HIV-1 set-point viral load below the mean and CMV acquisition after 1 month *others* = children with high set-point viral load or early CMV acquisition (Groups 1, 2, and 3 from the previous table). ^amean survival times are restricted to the follow-up of 24 months, children not dying before 24 months are censored, so true mean is underestimated. ^b No children with low HIV-1 set-point and late CMV acquisition died, all data are censored so no mean survival time can be estimated.

Discussion

Although the burden of CMV disease has been substantially relieved by the advent of HAART in 1996(182, 255, 408), CMV retinitis and end organ disease continue to be major causes of morbidity in HIV-1 patients(39, 125, 126, 570, 571, 584). CMV is not quiescent in patients on HAART with good HIV-1 suppression and even low levels of CMV viraemia have been associated with increased disease progression and mortality(126, 159). CMV has been associated with increased mortality among HIV+ infants, though the mechanism by which CMV accelerates HIV-1 disease is unknown. In this Kenyan paediatric study of HIV-1 infected and uninfected infants, CMV prevalence was extremely high during early infancy. Infants with HIV-1 contained CMV less effectively than HIV-1 uninfected infants and HIV-1 and CMV levels were correlated. The timing of CMV acquisition was found to have a significant effect on survival during the first year of life in co-infected infants.

Because this study was conducted retrospectively and only cryopreserved plasma specimens were available for diagnosis and quantification of CMV, these data must be interpreted with the following limitations in mind. Firstly, levels of CMV DNA in plasma are lower than in cells and the figures reported here likely underestimated true levels of viraemia. However, since comparisons of HIV-1 infected and uninfected infants were made using identical methods the differences between groups could be discerned despite some decreased sensitivity in the assays. In addition, since >95% of the infants in the study had detectable viraemia at some point during follow-up, it is unlikely that false-negatives contributed significantly to overall prevalence estimates, though they may slightly modify estimates of timing of CMV infection and point-prevalence. A second limitation of the study was that the cohort was not primarily

established to study CMV and was not designed to identify clinical signs of CMV infection. No CMV symptoms were detected in the congenitally infected infants in this study, which is consistent with previous reports showing children born to chronically infected women are often asymptomatic(519). CMV rarely causes clinical symptoms when viral loads are low, and the levels of CMV viraemia detected in the current study suggest that CMV is unlikely to be the direct cause of death in the infants(306) (553). However, paediatric CMV and HIV-1 can contribute to several non-specific symptoms such as failure to thrive, sepsis, and gastrointestinal disease; these were morbidities that significantly contributed to mortality in the study cohort(401) and it is possible CMV disease played a role in some of these deaths. Finally, HIVexp- provided a control group for the CMV studies. These infants are not ideal controls as “healthy infants”, as they have an increased rate of mortality during the first year of life and altered immune function compared to infants born to HIV-1 negative mothers(104, 205).

CMV incidence was very high during the first year of life; more than 80% of infants were infected by 3 months of age, independent of HIV-1 infection status. This figure is higher than has been reported in The Gambia (50-60% CMV prevalence at 6 months and ~85% at 12 months of life) (40) (553). CMV is acquired vertically (*in utero* or via breast milk) and horizontally (from other children, relatives)(140, 227, 517), and the very high prevalence in this cohort is likely due to a combination of mixed modes of transmission.

Congenital CMV occurred in a high proportion of infants who became infected with HIV-1 *in utero*. This suggests similar maternal-infant factors may influence

susceptibility to HIV-1 and CMV infection *in utero*. Increased risk of congenital CMV infection has been previously noted in HIV-1 infected children(149). Women with cervical shedding of HIV-1 are more likely to be shedding CMV and this may contribute to an increased risk of intra-partum transmission (380). Breast milk viral load is correlated with transmission in both CMV (227) and HIV-1 (454). No correlation was found between maternal CMV viraemia and congenital CMV transmission in this cohort. There are other reports of discordance between maternal viraemia and congenital CMV transmission, and anti-IgG avidity tests are more predictive of transmission(311, 441). Congenital CMV typically results from primary infection during pregnancy(518) but it is unlikely that the 5 women who transmitted CMV *in utero* were acutely infected, as they were CMV seropositive at delivery and had low CMV viral loads. Discordance between maternal viraemia and congenital transmission may indicate that CMV replication at localized sites during chronic infection is sufficient for transmission despite a lack of systemic reactivation.

CMV viral load was examined during the first year of life in infants with and without HIV-1 co-infection. CMV levels were highest among children with early acquisition of HIV-1, suggesting acute co-infection with HIV-1 compromised containment of CMV. CMV levels were similar among children with late HIV-1 acquisition and those who remained uninfected. Longitudinal comparisons of viral load over time showed more rapid decline of CMV viral load in HIVexp- infants than in the HIV+ group. HIV-1 facilitates CMV replication via systemic immunosuppression, and this is likely to explain the differences in kinetics of CMV replication in HIV-1 infected and exposed uninfected infants(151, 175, 254).

A previous study by Boriskin et al. reported higher levels of CMV viral load in infants < 2 years of age with advanced disease progression, but found no correlation between cellular CMV load and HIV-1 viral load (60). The suppressive effect of ARVs in the Boriskin study is likely explain the differences in findings. Several mechanisms may explain the link between HIV-1 and CMV viral replication. First, CMV is an opportunistic pathogen that replicates more easily in immunosuppressed individuals(151, 175, 254); high HIV-1 viral load and immunosuppression is likely to translate to impaired control of CMV replication. Second, HIV-1 and CMV may interact directly at the single cell level, either through transactivation of HIV-1 via its promoter or by altering HIV-1 tropism(218, 308). Though the co-factor effect has been demonstrated *in vitro*, there is yet no evidence this occurs naturally during infection, and since HIV-1 and CMV establish their reservoirs in different cell types, an indirect mechanism seems more likely. Finally, CMV may augment HIV-1 replication via widespread cellular activation. HIV-1 requires cellular activation for efficient infection of T cells(146). Monocytes are productively infected by CMV,(234) and through production of IL-12 and their role as antigen presenting cells, have the potential to activate CD4 T cells.

CMV viral load has previously been reported to predict survival in adults with HIV-1 infection (126, 159, 510). Peak CMV viral load was only predictive of death in the children with *in utero* acquisition of HIV-1. After adjusting for HIV-1 set-point, no independent effect of CMV viral load was detected. The strong correlation between HIV-1 and CMV viral load further confounds the analysis, and if the level of CMV viraemia contributes independently to mortality, a larger study would be necessary to see an effect of CMV viral load over that of HIV-1 viral load.

Others have reported poor prognosis for HIV-1 infected children born with congenital CMV (149). In the current study, 3 of the 4 children with HIV-1 and CMV co-infection at birth died. Though the frequency of congenital CMV transmissions was high in the current study, the numbers were not large enough to perform a similar analysis examining congenital co-infection and disease progression. However, it is likely that the true number of congenital infections was underestimated in the current study, as discussed above, the sensitivity of plasma viraemia has not been evaluated in infants as a diagnostic method. All infections before 1 month were thus grouped together to examine the effect of early CMV acquisition on mortality. Among all children with co-infection, early CMV was associated with a trend in increased mortality at 24 months. No mortality was observed in the group with low HIV-1 set-point and CMV acquisition after 1 month, suggesting that delaying CMV acquisition may benefit survival in children with low HIV-1 viral load. Timing of CMV acquisition had no effect on mortality in children with high set-point HIV-1.

CMV remains the most common viral cause of congenital disease globally(477, 478) and even among healthy children presents significant morbidity and developmental defects. CMV diagnostics among asymptomatic newborns is not standard practice for the care of healthy or HIV-1 infected infants, and the value of treating sub-clinical CMV viraemia to support HIV-1 treatment has not been investigated in neonates. CMV treatment is invasive, toxic and expensive(47), and may not be practicable considering the current scale of the HIV-1 epidemic. However, screening for CMV by PCR or antigenaemia may identify HIV-1 infected children at especially high risk for mortality. Several studies have shown CMV prophylaxis with oral ganciclovir or

valaciclovir to reduce morbidity and mortality in immunosuppressed patients (125, 511) (159). The importance of a CMV vaccine, and better therapeutic agents for the treatment of CMV should be re-assessed in context of the paediatric HIV-1 pandemic. Though HAART will undoubtedly change the epidemiology of CMV infection in paediatric populations, the global community may be years away from the WHO's target of universal access to ARV treatment. Until that goal is realized, a CMV vaccine may have great benefit for children with undiagnosed HIV-1 infection.

Summary

CMV prevalence is high among Kenyan children exposed to HIV-1 infection. HIV-1 infection impaired containment of CMV replication. CMV infection contributes to HIV-1 disease progression co-infected children, particularly those with concurrent acute infection.

CHAPTER 4

Lymphocyte distributions in healthy and HIV-1 exposed Kenyan infants

Introduction

The understanding of viral infections in young infants may be confounded by the complexity of factors influencing the development of immunity in early life. Age-related changes in T cell subsets, first exposure to environmental pathogens, and vaccination may result in the diversification of lymphocyte populations. In Chapter 3 CMV incidence was shown to be very high during the first months of life in Kenyan infants born to HIV-1 infected mothers. CMV infection in particular has been shown to elicit dramatic changes in the CD8 cell subset that can persist throughout life (372, 417, 572, 578). CMV may play an important role in the shaping of lymphocyte populations in Kenyan children.

The aim of this chapter is to establish age-normal values for cellular phenotype in Kenyan children who are at high risk for CMV infection. Two groups of infants are studied, drawn from a cohort of HIV-1 negative women and children (MIP cohort-children unexposed to HIV-1) and HIV-1 positive mothers (CTL cohort-infants exposed to HIV-1). The second aim of this chapter is to assess CMV-induced changes in lymphocyte phenotype, and assess the relationship between cellular activation and CMV viral load.

Materials & Methods

Subjects & sampling

The CTL cohort was described in Chapter 2. CMV and HIV-1 diagnostics were described in Chapter 3. Ten HIV-1 exposed uninfected children (HIVexp-) were originally selected based on survival to 12 months and availability of specimens, but specimens from 3 children did not arrive from Kenya, so in total 7 HIVexp- children were followed up longitudinally. One additional child with a single specimen stored at Oxford was screened at 6 months (CTL-B1-087).

Infants from a separate cohort of healthy HIV-1 negative women and their infants were also screened as HIV-1 unexposed controls. The MIP cohort was recruited in Nairobi in the same clinics as the CTL cohort. Following birth, infant HIV-1 status was further confirmed by PCR. MIP infants were not screened longitudinally, infants had blood collected at birth and one other time point post partum, at either 1, 3, or 6 month of age. Ten MIP infants were randomly selected for immunophenotyping studies.

In total, 18 HIV uninfected children were studied. MIP and CTL infants had different study follow-up. Children from the CTL group (born to HIV-1 infected mothers) were screened serially during the first year of life (birth, followed by 1, 3, 6, 9, 12 months) according to sample availability. CMV viral loads were performed on MIP infants as described in Chapter 3. However, because of larger gaps in sampling it was not possible to estimate the timing of CMV acquisition as precisely in the MIP infants as it was in the HIVexp- infants.

Specimen collection

Venous and cord blood was collected into EDTA tubes. Approximately 0.2 – 3 ml of blood were collected from each infant. Two 7-ml tubes of cord blood were collected immediately after delivery. Whole blood was centrifuged for 10 minutes at 1500 rpm to separate plasma, which was divided into aliquots and stored at -80°C . The remaining blood was then diluted with equal volume of RPMI medium, separated by density gradient centrifugation, and washed with RPMI cell media supplemented with 10% FCS (R10). Cells not used in fresh immunological assays were cryopreserved in FCS supplemented with 10% DMSO freezing media and stored in liquid nitrogen. Between 2 and 10 million PBMC were frozen in each vial.

Multicolour flow cytometry

Cryopreserved PBMC were thawed at 37°C and placed into 5 ml of sterile filtered FCS then washed twice with RPMI + 10% FCS (R10) before staining. Staining with Trypan blue indicated excellent viability (typically $>90\%$) though in most vials less than 100% of stored cells were recovered. Between 2-8 million cells were recovered per vial, and these were divided equally between wells for staining.

The phenotype of T cells was determined by the expression of cellular antigens using multicolour flow cytometry. Antibodies used in the study are shown in Table 4.1, and the mixed panels used in each stain are shown in Table 4.2. The antibody titrations, optimisations, and validation experiments involved in establishing the multicolour flow assays are described in detail in Chapter 8. To minimise intra-patient variability all cells from a single infant were stained in the same experiment. Cells were spun

down and supernatant removed leaving a minimal volume (approximately 20 μ l) of media. To minimise intra-assay variability antibody master-mixes were made in each experiment and the aliquots were added to cells using a multi-channel pipette. Since small cell numbers were expected from some of the cryopreserved specimens, staining was performed in 96-well V-bottom tissue culture plates to maximise cell adherence during washing steps. This method also reduced pipetting steps and the volume of reagents used. Cells were incubated with antibody for 20 minutes in the dark at room temperature, washed twice with PBE (PBS plus 0.5% BSA; 0.5 mM EDTA) and resuspended in CellFix solution (BD Pharmingen, Oxford, UK). The volume of CellFix diluent was determined by the approximate number of cells in each tube, this was typically 170-200 μ l.

For Panels B and D, the above protocol was followed for surface staining. Instead of the final fixing step, cells were permeabilised by adding 300 μ l of CytoPerm/CytoFix permeabilisation solution (Pharmingen) and incubating for 20 minutes in the dark at 4 °C. Cells were washed twice with Perm buffer, which uses saponin to permabilise the cells. Since saponin permeabilisation is reversible, the remaining wash and incubation steps were done in the Perm buffer. Cells were stained with intracellular antibodies in a volume of 100 μ l for 30 minutes in the dark at 4 °C, washed twice and resuspended in 170-200 μ l of buffer.

Excluded assays

Due to procedural errors, some infants had incomplete antibody panels: MIP-B1-110 and MIP-B1-122 do not have Bcl-2 or perforin staining (panels B&D). Upon thawing, the majority of cells from MIP-B1-173 and MIP-B1-167 were dead; these

assays are excluded from the analysis. The perforin-PE staining was not consistent in staining patterns, and did not match previous reports, and is excluded from analysis here.

Analysis and gating

Cells were acquired on a Cyan ADP or LX instrument using Summit software (Dako Cytomation, Angel Drove, UK). As many cells as possible were acquired in each tube. Cells were acquired at 1500 to 3000 events/second. This was achieved by diluting the cells with PBS and adjusting the flow stream. Flow stream adjustments were kept to the minimal level to prevent the frequency of “doublets” (E.g., more than one cell) passing through the laser at once. FCS files were analysed with FlowJo software (Treestar, Inc., Olten Switzerland). Data were re-compensated during analysis using single stain controls and FlowJo’s automated compensation platform. Cells stained with isotype controls were used to establish cut-off channels for positive and negative staining. A single donor’s cryopreserved cells provided all compensation and isotype controls for the experiments so antibody stability and instrument variability could be monitored over time.

The major T cell subsets were defined as shown in Figure 4.1. First, granulated cells, dead cells and debris were excluded by size and granularity. Gating of lymphocytes was extended to high forward scatter to include larger, activated cells. Next, CD3 expression was used to exclude monocytes and NK cells. The expression of high levels of CD4 or CD8 was used to distinguish the major T cell subsets. In neonates, a large number of CD4+CD8+ T cells were observed. These cells are likely to be a mix

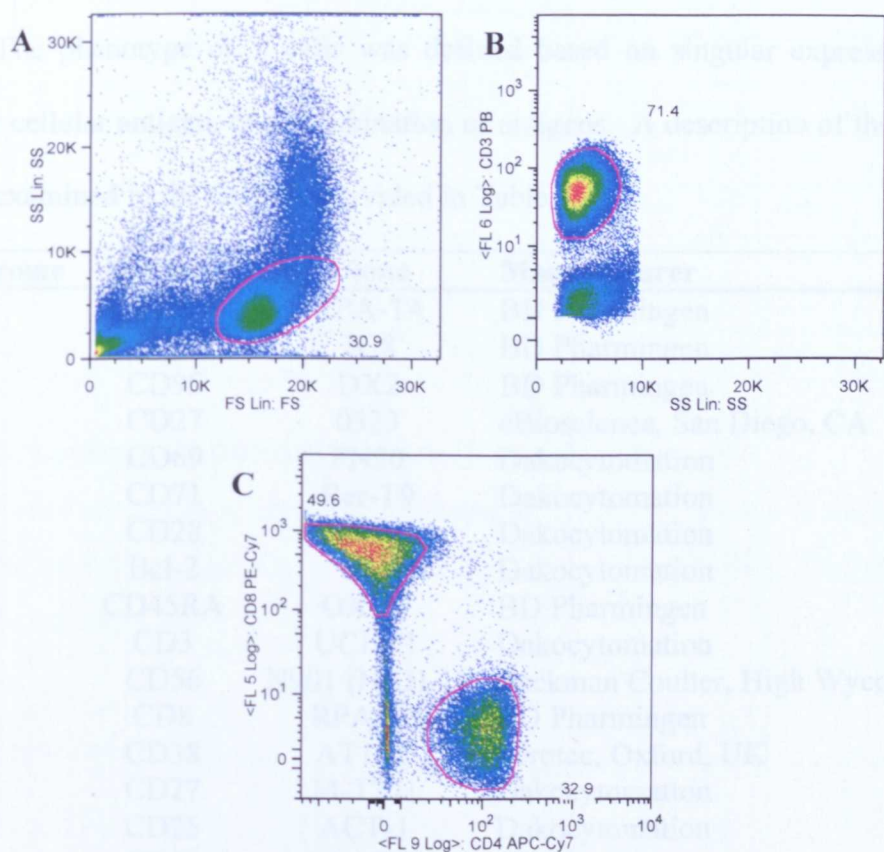


Figure 4.1. Gating to select CD4+ and CD8+ T cell subsets.

A) Exclusion of debris, dead cells and granulated cells. B) Selection of T cells by CD3+ expression. C) Selection of pure single positive CD8bright and CD4+ cells.

of recent thymic emigrants, activated CD4 and CD8 cells. These cells were excluded for the current analysis to maximize purity of CD4 and CD8 single-positive T cell subsets. The phenotype of T cells was defined based on singular expression of a particular cellular antigen, or a combination of antigens. A description of the cellular antigens examined in the study is provided in Table 4.3.

Fluorochrome	Antibody	Clone	Manufacturer
AC7	CD4	RPA-T4	BD Pharmingen
AC7	CD16	3G8	BD Pharmingen
APC	CD95	DX2	BD Pharmingen
APC	CD27	0323	eBioscience, San Diego, CA
FITC	CD69	FN50	Dakocytomation
FITC	CD71	Ber-T9	Dakocytomation
FITC	CD28	CD28.1	Dakocytomation
FITC	Bcl-2	124	Dakocytomation
FITC	CD45RA	OX-33	BD Pharmingen
PB	CD3	UCHT1	Dakocytomation
PC5	CD56	N901 (NKH-1)	Beckman Coulter, High Wycombe UK
PC7	CD8	RPA-T8	BD Pharmingen
PE	CD38	AT13/5	Serotec, Oxford, UK
PE	CD27	M-T271	Dakocytomation
PE	CD25	ACT-1	Dakocytomation
APC	CD71	M-A712	BD Pharmingen
PC5	CD152	BNI3	BD Pharmingen
PE	CCR7	FAB197	R&D Systems, Minneapolis, MN
APC	CD45RA	HI100	BD Pharmingen
APC	HLA-DR	TU36	BD Pharmingen
PE	perforin	27-35	BD Pharmingen
FITC	CD57	TB01	Serotec

Table 4.1. Antibodies used in immunophenotyping studies.

Fluorochrome	A Co-stimulation	B Apoptosis	C Proliferation Homing & Senescence	D Cytotoxicity	E Activation	F Treg
FITC	CD28	Bcl-2	CD57	CD28	CD69	CD71
PE	CD27	CD38	CCR7	Perforin	CD38	CD25
PE-Cy5	CD56	CD56	CD56	CD56	CD56	CD152
PE-Cy7	CD8	CD8	CD8	CD8	CD8	CD8
<i>Pacific Blue</i>	CD3	CD3	CD3	CD3	CD3	CD3
APC	CD45RA	CD95	CD71	CD27	HLA-DR	CD45RA
APC-Cy7	CD4	CD4	CD4	CD4	CD4	CD4

Table 4.2. Antibody panels used to describe cell phenotype.

Fluorochromes are shown in the left most column. Labels at the top of each column describe the main phenotype described by each mixture of antibodies. Primary subset markers are shown in bold.

Data management

Flow cytometry data was exported from FlowJo as text files, modified with a program and uploaded into a customised database. Queries were used to extract flow cytometry data, viral loads, and infant mortality data. Though several hundreds of different analysis possibilities (cell phenotypes) were imported into the database, only a small fraction of these were extracted for analysis here.

Statistical methods

Clinical and laboratory data were collected and stored in a Microsoft Access Database (Microsoft Inc., Redmond, Washington, USA) and analysed using Stata SE v.9 (Stata Corp. College Station, Texas, USA). Cell frequencies and viral loads were log₁₀-transformed before comparisons. Mann-Whitney rank-sum tests were used to compare

median cell frequencies between MIP and HIVexp- groups of infants. Comparisons of frequencies between visits were pair-wise with matched values for each infant. Correlations were tested using Spearman’s rank test. Models of cell frequencies and viral load over time were generated using cubic smoothing splines of time-point medians.

Antigen	Phenotype defined
CD3+	<i>T cell</i>
CD4+	<i>CD4 subset</i>
CD8 ^{bright}	<i>CD8 subset</i>
CD25+	<i>IL-2R: utilisation of IL-2, activation, Treg (high on CD4+)</i>
CD27/CD28	<i>Early (CD27+CD28+) and late (C27-CD28-) effector phenotype of CD4 or CD8. Intermediate CD4 (CD27-CD28+), intermediate CD8 (CD27+CD28-)</i>
CD45RA+/-	<i>Memory phenotype, transient expression from CD45RA+ → CD45RA- → CD45RA+</i>
CD152+	<i>CTLA-4: negative regulatory signalling</i>
CD57+	<i>Senescence</i>
CCR7+	<i>Homing to lymphatics, antigenic naivety</i>
CD71+	<i>Transferrin receptor: iron utilization, proliferation</i>
CD38 ^{bright}	<i>Constitutive expression on neonatal T cells, activation with co-expression of HLA-DR</i>
CD69+	<i>Early lymphocyte activation</i>
HLA-DR+	<i>Class II MHC: activated T cells</i>
CD95+	<i>FasR: vulnerability to apoptosis via Fas pathway, on Bcl-2^{dim} cells highly vulnerable to apoptosis</i>
Bcl-2 ^{bright}	<i>Protection from apoptosis</i>
Perforin	<i>Component of granule-mediated cytotoxicity</i>

Table 4.3. Lymphocyte subsets examined in the study.

Infant	Month CMV detected	Follow-up
HIV-unexposed		
MIP-B1-104	aviraemic	M6
MIP-B1-110	birth	CBMC & M6
MIP-B1-111	6	CBMC & M6
MIP-B1-119	aviraemic	CBMC & M6
MIP-B1-122	aviraemic	CBMC & M6
MIP-B1-134	aviraemic	CBMC & M1
MIP-B1-137	aviraemic	CBMC & M1
MIP-B1-146	aviraemic	CBMC & M1
MIP-B1-167	3	CBMC & M3
MIP-B1-173	aviraemic	M3
HIV-1 exposed uninfected		
CTL-B1-059	3	M1-12
CTL- B1-081	3	CBMC-M12
CTL- B1-087	NT	M6
CTL- B1-103	aviraemic	CBMC & M1
CTL- B1-122	3	CBMC-M12
CTL- B1-180	3	M1-12
CTL- B1-272	3	CBMC-M15
CTL- B1-461	3	CBMC-M15

Table 4.4. Summary of follow-up and CMV detection in HIV-uninfected children.

MIP children were born to HIV-1 negative mothers. CTL children were born to HIV-1 positive mothers, but remained HIV-1 negative by DNA and RNA assays for the duration for study follow up. NT = not tested, CBMC = cord blood mononuclear cells, M=month of study follow-up.

Results

Lymphocyte distribution in healthy Kenyan infants from the MIP cohort

Table 4 summarises the follow-up and CMV testing in the children. Frequencies of cells with selected phenotypes are shown for HIV-1 uninfected MIP infants in Table 5 (CD4+) and Table 6 (CD8+). Though there were specimens examined at 1 and 3 months, this was a very small number of infants (4 children) so only data from birth and 6 months are shown. At birth, both CD4 and CD8 subsets consisted of primarily resting, non-activated, naïve cells. In cord blood, the majority of CD4 and CD8 T cells were CD27+CD28+, CD45RA+, CCR7+, indicating a naïve phenotype. The frequency of cells expressing CD152 (CTLA-4) was also low in both CD4 and CD8 subsets. Overall, frequencies of activated cells were low, as measured by the expression of CD69+, HLA-DR+, or CD38^{bright}HLA-DR+ in both the CD4 and CD8 subsets. The majority of cells expressed CD38 at high levels, consistent with other reports in newborns. In the cord blood low frequencies of cells expressed markers indicating vulnerability to apoptosis, CD95+ frequencies were low and >99% of cells expressed high levels of Bcl-2. CD71 expression was used to estimate the frequency of proliferating cells. It was expressed on approximately 1% of cord blood CD4 and CD8. Very low frequencies of senescent (CD57+) cells were observed in the cord blood, <2% in both CD4 and CD8 subsets. Approximately 4% of CD4 T cells expressed high levels of CD25+ consistent with a regulatory T cell phenotype.

Only 3 infants provided matched data at birth and 6 months, so pair-wise comparisons were underpowered and could be misleading. It is not possible here to measure with precision differences between the 6 month and cord blood frequencies, but the data is

presented here for reference, and for later (non pair-wise) comparison with HIVexp-infants. Though a small number of infants were examined at 6 months of age, there was evidence of antigenic stimulation in CD4 and CD8 T cells, particularly in the CD8 subset. At 6 months, approximately 40% of CD4 and 50% of CD8 had lost expression of CCR7. Evidence of antigenic stimulation was also detected by diversification of CD27CD28 subsets from CD27+CD28+ into double single, and double negative populations in the CD4 and CD8. CD45RA+ and CD45RO-subpopulations were diverse in CD27CD28 expression, indicating the outgrowth of effector cells. CD71+, CD152+ and Treg frequencies remained low at 6 months. Overall, frequencies of activated T cells were low at 6 months in both CD4 and CD8 subsets (<10% were CD69+, HLA-DR+, or CD38^{bright}HLA-DR+), though the range of expression in these markers was wide; some infants had high frequencies of activated cells (up to 13% CD4+CD38^{bright}HLA-DR+, and 26% CD8+CD38^{bright}HLA-DR+). At 6 months cells highly vulnerable to apoptosis (Bcl-2^{dim}CD95+) had appeared (median 4% of CD4, 10% of CD8), though the majority of cells continued to express the highly protected (Bcl-2^{bright}CD95-) phenotype (median 62% of CD4, 63% of CD8). CD57+ cells began to appear at high frequencies in the CD8 subset (median 20% in CD8) but remained a very small population in the CD4 subset (median 1.3%). Overall, the phenotype of cells at 6 months is representative of a diversifying population of T cells, though it is not possible to know from the current data if this is due to age, infection, vaccination, or some other unmeasured environmental influence.

	cord blood (n=8)	6 months (n=4)
Co-stimulation		
Early (CD27+CD28+)	89.9 (64.1-94.3)	71.4 (28.5-93.5)
Intermediate (CD27-CD28+)	3.81 (0.62-28.3)	18.2 (1.92-30)
Late (CD27-CD28-)	1.96 (1.13-9.19)	5.11 (0.74-49.4)
CD45RA+	72.7 (25.7-81.9)	50.8 (31.4-69.7)
^a CD45RA+/Early	92.5 (77.0-94.4)	81.4 (38.4-94.6)
CD45RA+/Intermediate	2.41 (0.58-17.9)	13.3 (1.04-19.9)
CD45RA+/Late	1.91 (0.83-7.1)	4.34 (0.61-39.6)
^a CD45RA-/Early	84.3 (60.2-94)	63.6 (24.2-92.5)
CD45RA-/Intermediate	7.00 (0.79-33.5)	21.7 (3.24-39.4)
CD45RA-/Late	2.24 (0.68-17.4)	5.98 (0.92-52.8)
CD152+	1.76 (0.68-3.72)	1.19 (0.82-8.1)
Vulnerability to apoptosis		
Bcl-2 ^{bright}	99.2 (93.9-99.7)	87.2 (79.7-94.7)
CD95+	14.2 (2.24-35.4)	30.0 (15.2-44.7)
Bcl-2 ^{bright} CD95-	77.0 (64.2-93.9)	62.0 (53.5-70.4)
Bcl-2 ^{dim} CD95+	0.72 (0.13-3.11)	3.73 (2.81-4.65)
Homing & proliferation		
CCR7+	84.1 (80.2-89)	61.2 (41.5-80.8)
CD57+	1.21 (0.79-1.26)	1.26 (1.23-1.29)
CD71+	0.965 (0.7-1.29)	0.89 (0.86-0.92)
Activation		
CD69+	1.51 (0.3-29.5)	0.995 (0.36-3.37)
HLA-DR+	2.94 (1.84-11.3)	5.66 (2.31-17.4)
^b CD38 ^{bright}	94.6 (84.8-98.8)	85.8 (79-96.1)
CD38 ^{bright} HLA-DR+	3.065 (1.75-10.8)	5.28 (2.22-13.2)
Treg	4.21 (1.49-8.35)	2.90 (0.57-7.41)

Table 4.5. CD4 lymphocyte distribution in healthy children born to HIV-negative women.

Median cell frequencies (min-max) are shown as percent of CD4 cells, except

^apercent of CD45RA+ or RA- cells. ^bCD38^{bright} cells are not all activated, CD38 is constitutively expressed on neonatal cells.

	Cord blood (n=8)	6 months (n=4)
Co-stimulation		
Early (CD27+CD28+)	63.8 (35.2-75)	31.4 (9.13-65.3)
Intermediate (CD27+CD28-)	29.4 (23.2-54.8)	36.2 (30.5-48.7)
Late (CD27-CD28-)	1.14 (0.1-19.6)	28.5 (0.36-52.6)
CD45RA+	85.5 (46.6-92.2)	61.4 (42.3-79.4)
^a CD45RA+/Early	68 (32.6-76.8)	42.5 (9.39-69.8)
CD45RA+/Intermediate	28.5 (21.3-50)	32.9 (24.6-47.2)
CD45RA+/Late	1.06 (0.032-37.3)	21.2 (0.11-54.8)
^a CD45RA-/Early	49.4 (21.7-78.6)	21.0 (8.18-44.3)
CD45RA-/Intermediate	49.65 (19.1-78.3)	49.2 (37.5-54.4)
CD45RA-/Late	0.44 (0-3.73)	34.1 (1.3-44.6)
CD152+	2.05 (0.95-7.26)	2.03 (1.01-6.72)
Vulnerability to apoptosis		
Bcl-2 ^{bright}	99.3 (97.9-100)	77.0 (59.5-94.4)
CD95+	5.98 (0.88-12.7)	25.4 (20.2-30.6)
Bcl-2 ^{bright} CD95-	89.7 (72.6-98.3)	63.3 (49.1-77.4)
Bcl-2 ^{dim} CD95+	0.179 (0-0.72)	10.1 (1.48-18.8)
Homing & proliferation		
CCR7+	80.5 (75.5-87.2)	50.75 (32.4-69.1)
CD57+	1.17 (1.07-1.88)	19.8 (4.4-35.2)
CD71+	1.085 (0.88-2.07)	0.665 (0.42-0.91)
Activation		
CD69+	2.09 (0.43-19.6)	2.07 (0.53-4.27)
HLA-DR+	3.32 (1.23-18.2)	7.26 (5.1-20.2)
^b CD38 ^{bright}	90.5 (65.1-94.6)	78.7 (52.4-92.9)
CD38 ^{bright} HLA-DR+	2.15 (1.18-18.7)	6.48 (4.82-25.8)

Table 4.6. CD8 lymphocyte distribution in healthy children born to HIV-negative women.

Median cell frequencies (min-max) are shown as percent of CD8 cells, except ^apercent of CD45RA+ or RA- cells. ^bCD38^{bright} cells are not all activated, CD38 is constitutively expressed on neonatal cells.

Lymphocyte distribution in HIV-1 negative children born to HIV-1 infected women

Phenotype and CMV viral load data were available for 8 HIV-1 exposed uninfected (HIVexp-) children. Lymphocyte distributions for selected cellular antigens are shown in Tables 4.7 and 4.8. Comparisons were made between MIP and HIVexp- phenotype distributions in cord blood and at 6 months. In cord blood, the HIVexp- infants were less diverse in CD4⁺ CD27CD28 subpopulations than the unexposed (MIP) infants. In cord blood, the unexposed MIP infants had higher frequencies of CD4⁺CD27⁻CD28⁻ compared to the HIVexp- group (2% vs 0.5%, $p=0.03$). The HIVexp- infants had lower frequencies of Bcl-2^{bright} cells (median 83% vs 99%, $p = 0.03$) than the unexposed infants. Frequencies of regulatory T cells were higher among the HIVexp- group than the unexposed MIP cord blood (9.6% vs 4.2%, $p = 0.02$).

In the CD8 subset, the unexposed MIP infants were also more diverse in CD27CD28 expression at baseline, with lower cord blood frequencies of CD27⁺CD28⁺ than the HIVexp- infants (64% vs 84%, $p = 0.007$) cells. Higher frequencies of CD8⁺Bcl-2^{bright} cells were present in the cord blood of unexposed infants compared to HIVexp- infants (99% vs 86%, $p = 0.01$). In the CD45RA⁺ subpopulation of CD8⁺, MIP children had more cells in the intermediate and late phenotypes compared to HIVexp- infants (intermediate 28.5% vs 13.7, $p = 0.007$, late 1.06% vs 0.061%, $p = 0.05$). In the CD45RA⁻ subset, MIP children had lower frequencies of early cells compared to HIVexp- (49.4% vs 76.2%, $p = 0.05$).

At 6 months, no differences in CD4 subpopulations were detected between the MIP and HIVexp- children. Higher frequencies of apoptotic-vulnerable CD8 cells were detected in the HIVexp- infants. CD8+CD95+ (37% vs 25%, $p = 0.05$) and CD8+Bcl-2^{dim}CD95+ (29% vs 10%, $p = 0.05$) cell frequencies were higher in the HIVexp- group.

	Cord blood (n=5)	1 month (n=6)	3 months (n=6)	6 months (n=5)	9 months (n=5)	12 months (n=6)
Co-stimulation						
Early (CD27+CD28+)	93.9 (79.8-99.4)	94.5 (91.2-98.2)	92.2 (87.9-96.3)	91.9 (83.8-94.8)	91.3 (87.4-94.9)	91.3 (80.8-95.5)
Intermediate (CD27-CD28+)	4.75 (0.26-16.3)	4.37 (0.48-7.24)	4.78 (0.42-8.79)	4.88 (0.76-10.6)	4.27 (1.03-11.1)	4.57 (1.48-14.7)
Late (CD27-CD28-)	0.51 (0.04-1.88)*	0.24 (0.11-1.34)	2.16 (0.24-3.76)	1.94 (0.48-4.85)	1.09 (1.04-3.44)	1.83 (0.26-3.44)
CD45RA+	57.7 (37.5-80.7)	65.5 (53.5-86.7)	77.8 (53.4-88.3)	81.9 (49.8-83.2)	67.7 (56.9-85.6)	69.6 (55.3-94.2)
^a CD45RA+/early	96.2 (81-99.6)	96.6 (94.1-98.7)	96.5 (90.4-97.6)	93.4 (89-97.4)	95.4 (93.6-97.7)	95.6 (87.4-97.9)
CD45RA+/intermediate	2.65 (0.15-14.8)	2.18 (0.16-3.29)	2.17 (0.16-6.29)	2.92 (0.2-6.55)	1.83 (0.32-2.94)	1.38 (0.23-8.12)
CD45RA+/late	0.42 (0.035-2.03) [‡]	0.23 (0.13-1.48)	1.1 (0.13-2.73)	1.39 (0.37-3.36)	0.85 (0.63-2.35)	1.27 (0.21-2.13)
^a CD45RA-/early	90.7 (77.9-99)	91.8 (82.8-97.3)	80.45 (74.6-93.9)	85.3 (73.2-92.2)	78.6 (71.4-91.1)	79.7 (71.5-91.5)
CD45RA-/intermediate	7.48 (0.49-18.5)	7.16 (1.11-16.7)	14.5 (1.37-17.7)	10.2 (3.43-18.5)	14.6 (2.7-26.7)	13.7 (4.72-23.4)
CD45RA-/late	0.65 (0.049-1.66)*	0.28 (0.085-1.16)	3.55 (0.64-9.96)	3.47 (0.98-12.2)	1.7 (1.48-7.11)	2.54 (0.39-8.78)
CD152	0.68 (0.2-0.86)	0.93 (0.44-1.29)	0.7 (0.22-1.8)	1.005 (0.72-1.45)	0.68 (0.47-1.1)	0.65 (0.32-1.61)
Vulnerability to apoptosis						
Bcl-2bright	82.7 (74.3-98.1)*	92.4 (77.2-96.7)	81.9 (55-94.8)	80 (73.7-90.1)	84.2 (72.7-93.4)	74.4 (62.4-94.5)
CD95+	16 (15.7-17)	16.3 (5.89-31.1)	22 (2.84-37)	13.1 (8.28-26.7)	13.6 (8.63-35.6)	19.3 (8.78-36.6)
Bcl-2brightCD95-	68.5 (65-84.4)	72 (68.1-87.1)	62.8 (53.3-73.5)	70.3 (66.7-80.8)	66.8 (61.3-76)	63.6 (54.2-77.2)
Bcl-2dimCD95+	2.39 (0.37-3.12)	2.86 (1.2-5.9)	4.19 (1.08-10.6)	3.92 (2.72-6.95)	4.7 (3.01-8.68)	6.29 (2.7-9.99)

Table 4.7. CD4 phenotypes in HIVexp- infants (continued on next page). Median cell frequencies (min-max) are shown as percent of CD4 cells, except [‡]percent of CD45RA+/- . #p = 0.02, *p = 0.03, [‡]p = 0.04 for comparison with MIP group.

	Cord blood	1 month	3 months	6 months	9 months	12 months
Homing & proliferation						
CCR7+	80.5 (43.1-94.7)	62.8 (53.2-95.6)	64.6 (35.1-98)	52.5 (35-92.7)	48.4 (9.53-94.9)	68.2 (3.08-94.5)
CD57+	1.16 (0.6-1.4)	1 (0.29-2.41)	2.74 (0.31-5.26)	2.64 (1.02-3.84)	3.055 (1.05-3.36)	2.53 (0.41-3.91)
CD71+	0.67 (0.51-2.47)	1.97 (0.85-3.65)	2.72 (0.4-9.17)	1.005 (0.79-3.88)	2.35 (0.44-4.72)	2.7 (0.67-72.1)
Activation						
CD69+	5.86 (0.97-7.64)	3.47 (0.3-11.2)	1.82 (0.31-8.6)	2.61 (0.12-4.99)	2.37 (0.29-5.15)	1.82 (0.41-5.25)
HLA-DR+	2.99 (0.45-3.66)	4.64 (1.64-7.33)	6.045 (1.47-9.69)	4.36 (1.32-6.89)	5.46 (2.48-6.98)	5.92 (1.69-29.3)
^b CD38 ^{bright}	94.6 (76.5-96.7)	92.2 (63.1-96.3)	84.2 (55.8-95.1)	83.9 (41-93)	66.8 (28.1-91.6)	73.3 (40-90.7)
CD38 ^{bright} HLA-DR+	1.97 (0.44-3.5)	3.26 (1.53-3.69)	4.59 (1.35-5.31)	2.47 (1.23-3.49)	2.63 (2.04-2.83)	3.095 (1.48-24.2)
^b Treg	9.58 (7.4-10.9)#	8.865 (7.16-9.8)	4.74 (0.83-9.66)	7.275 (3.76-9.76)	5.53 (3.26-9.78)	6.8 (1.74-11.1)

Table 4.7. CD4 phenotypes in HIVexp- infants Median cell frequencies (min-max) are shown as percent of CD4 cells. #p = 0.02, *p = 0.03, \$p = 0.04 for comparison with MIP group. ^bNot all CD38^{bright} are activated, CD38 is constitutively expressed on neonatal T cells.

Cord blood		1 month	3 months	6 months	9 months	12 months
(n=5)		(n=6)	(n=6)	(n=5)	(n=5)	(n=6)
Maturation						
Early (CD27+CD28+)		84.3 (78.2-90.4)§	43.4 (23.3-56.5)	48.3 (33.8-53.9)	43.2 (16.7-51.2)	34.8 (15.7-63.6)
Intermediate (CD27+CD28-)		15.6 (9.59-21.7)§	44.6 (27.8-52)	31.6 (17.6-35.7)	17.1 (14.5-30.5)	26.6 (11.9-71.4)
Late (CD27-CD28-)		0.11 (0.019-0.14)	0.099 (0.00903-3.65)	26.2 (17.7-41.3)	33 (25.7-65.3)	35.8 (0.32-62.7)
CD45RA +		76.7 (66.2-94.4)	83.1 (76.9-96.4)	79.3 (63.9-85)	71.5 (65.9-89.3)	69.5 (64-84.1)
*CD45RA +/early		86.25 (79.8-91)§	88.3 (64.5-90.2)	52.2 (40.7-68.6)	52.7 (18.1-62.3)	42.8 (18.2-66)
CD45RA +/intermediate		13.68 (9-20.1)§	11 (7.96-35.4)	28.8 (11-31.7)	15.6 (9.69-30.7)	22.95 (7.93-72.8)
CD45RA +/late		0.061 (0.019-0.13)»	0.068 (0.00544-3.77)	19.9 (15-37.4)	27.6 (20.4-66.6)	33.8 (0.32-62.7)
<hr/>						
*CD45RA -/early		76.2 (51.9-85.4)»	16.2 (13.7-25.3)	20.9 (8.13-27.8)	21.2 (11.5-25.3)	17.6 (10.7-44.5)
CD45RA -/intermediate		23.6 (14.6-47.8)	61.6 (52.3-66.3)	40 (28.6-49.3)	26.6 (22.3-45.9)	36.1 (20-64)
CD45RA -/late		0.265 (0.018-0.36)	19.7 (8.39-31.3)	35.3 (29-63.2)	49.4 (31.9-64.7)	39.8 (0.37-67.4)
CD152		4.195 (0.39-7.09)	8.75 (0.61-17.5)	0.705 (0.31-5.58)	1.71 (0.051-4.01)	1.4 (0.063-6.27)
<hr/>						
Vulnerability to apoptosis						
Bcl-2bright		86.3 (68.1-97.5)‡	89.9 (88.7-96.1)	57.2 (29-65.8)	53.9 (33.2-65.5)	54.3 (29.2-68.9)
CD95+		7.55 (2.9-10.9)	18.5 (5.92-24.2)	36.7 (28.6-46.4)»	36.5 (32.1-67.7)	50.9 (19-56.1)
Bcl-2brightCD95-		84.05 (61-86.8)	80.5 (73.7-87.6)	50.7 (24.8-57.8)	46.9 (17.6-59.2)	36.7 (24.8-62.3)
Bcl-2dimCD95+		0.775 (0.51-2.36)	2.44 (2-4.03)	28.5 (20.7-40.7)»	25.5 (22.3-45.3)	27.8 (12.4-47.7)

Table 4.8. CD8 phenotypes in HIVexp- infants (continued on next page). Median cell frequencies (min-max) are shown as percent of CD8 cells, except ^apercent of CD45RA+/- cells. §p = 0.007, ‡p = 0.01, »p = 0.05 for comparison with MIP group.

	Cord blood	1 month	3 months	6 months	9 months	12 months
Homing & proliferation						
CCR7+	80 (67.2-93.6)	70.9 (65.9-89.5)	40.5 (21.2-87.6)	43.8 (29.5-56.9)	27.45 (14.3-59)	42.2 (10.8-99.3)
CD57+	2.16 (0.66-2.59)	2.33 (0.43-5.61)	41.7 (11.5-43.8)	35.6 (27.8-49.2)	41.4 (32.5-73.1)	50.6 (13.9-70.5)
CD71+	1.42 (0.32-6.47)	2.18 (0.39-4.19)	3.46 (0.33-8.88)	3.32 (0.72-4.94)	2.19 (0.55-3.51)	2.15 (0.88-3.16)
Activation						
CD69+	8.64 (5.9-11.7)	6.12 (0.75-11.8)	4.65 (0.87-16.4)	5.7 (0.76-9.53)	5.74 (0.58-8.59)	6.69 (0.76-9.86)
HLA-DR+	2.50 (1.2-3.34)	3.26 (1.5-7.94)	21.6 (5.59-42.1)	9.39 (5.14-27.2)	8.5 (6.35-19.4)	14.2 (5.04-20.4)
^b CD38 ^{bright}	82.4 (46.7-92.3)	84.4 (43.6-98.2)	81.05 (53.7-95.5)	67.2 (34.4-91.1)	56.1 (19.4-91.1)	63 (20.9-95.1)
CD38 ^{bright} HLA-DR+	1.78 (1.15-2.84)	3.18 (1.44-6.58)	20.4 (4.49-39.9)	7.69 (4.57-15.4)	7.18 (4.83-14.5)	9.34 (4.86-20.1)

Table 4.8. CD8 phenotypes in HIVexp- infants.

Median cell frequencies (min-max) are shown as percent of CD8 cells. §p = 0.007, ‡p = 0.01, »p = 0.05 for comparison with MIP group. ^bNot all CD38^{bright} are activated, CD38 is constitutively expressed on neonatal T cells.

CMV initiates changes in lymphocyte distributions from HIVexp- children

In the present cohort, CMV was highly prevalent among the HIV-1 uninfected children, 95% were viraemic by 12 months. To clearly delineate CMV- induced effects from age- induced effects on the immune system, a subgroup of 6 HIVexp- children were studied who had first become detectably CMV viraemic at 3 months of age. Pair-wise comparisons were made between frequencies at 1 month (pre-CMV) and 3 months (acute infection) and between 1 month and 12 months (chronic infection) for selected phenotypic markers.

Costimulatory phenotype

The expansion of late CD27-CD28- CD8 T cells has been described in healthy adults and children with CMV. Figure 4.2 shows an example of lymphocyte distributions during acute CMV infection in an HIVexp- infant. CTL-B1-081 became CMV viraemic at 3 months, with a baseline plasma viral load of 5021 CMV copies/ml. The first detection of CMV coincided with an increase in intermediate (CD27+CD28-) and late (CD27-CD28-) CD8 T cells, which by 12 months had become the predominant phenotype (62% of CD8). This pattern was observed in all of the infants with CMV viraemia (Figure 4.2 C&D). In the CD4 subset the increase in late phenotype cells was smaller in magnitude, but also significant ($p = 0.05$) during acute CMV. Late phenotype cells persisted during the period of study follow-up, with increased frequencies of late CD4 and CD8 at 12 months compared to pre-CMV levels at 1 month ($p = 0.04$ for CD4 subset, $p = 0.04$ for CD8 subset).

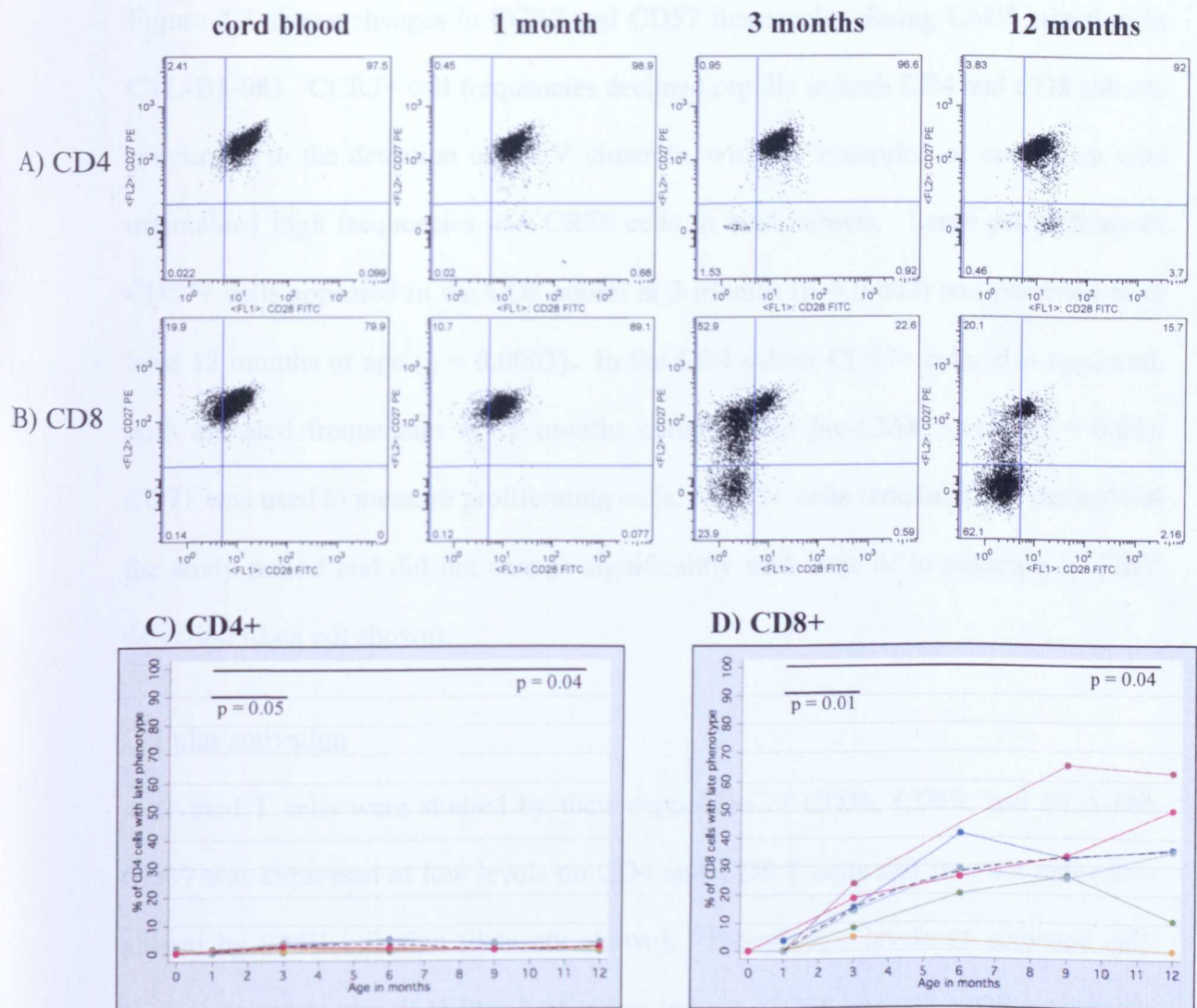


Figure 4.2. CMV infection initiates changes in co-stimulatory phenotype.

Dot plots show cells from HIVexp- infant CTL-B1-081 during the first year of life. Cells were stained with antibody panel A, 5000 events are shown for A) CD4⁺ and B) CD8⁺ T cell subsets. The patient became viraemic for CMV at 3 months of age with a viral load of 5021 copies/ml. C&D) show changes in late phenotype cell frequencies during the first year of life for all HIVexp- children with first CMV detection at 3 months. Coloured lines show data for individual patients, black dashed line is a median spline curve summarising all HIVexp- infants with first CMV at 3 months. P values indicate comparisons of month 1 vs 3, and month 1 vs 12.

Homing and senescence

CCR7 and CD57 were used to identify lymphoid homing and senescent T cells. Figure 4.3 shows changes in CCR7 and CD57 frequencies during CMV infection in CTL-B1-081. CCR7⁺ cell frequencies declined rapidly in both CD4 and CD8 subsets concurrent to the detection of CMV viraemia, with the exception of one infant who maintained high frequencies of CCR7⁺ cells in both subsets. Large populations of CD57⁺ cells appeared in the CD8 subset at 3 months ($p = 0.002$) and persisted to at least 12 months of age ($p = 0.0003$). In the CD4 subset CD57⁺ cells also appeared, with elevated frequencies at 12 months compared to pre-CMV levels ($p = 0.05$). CD71 was used to measure proliferating cells. CD71⁺ cells remained low throughout the study period and did not change significantly with time or in response to CMV detection (data not shown).

Cellular activation

Activated T cells were studied by their expression of CD38, CD69, and HLA-DR. CD69 was expressed at low levels on CD4 and CD8 T cells and was not detectably altered by CMV infection (data not shown). Though low levels of activated cells were detected in the cord blood of many infants, CD38 and HLA-DR expression changed dramatically following CMV detection. Figure 4.4 shows CD38 and HLA-DR staining in HEPS infant CTL-B1-081. A dramatic increase in CD38^{bright}HLA-DR⁺ cells is observed in the CD8⁺ subset at 3 months. Changes were more subtle in the CD4 subset, the frequency of activated CD4 T cells slowly increased in this child. Overall, activation peaked at 3 months then declined in the CD8 subset. At 12 months, frequencies of CD38^{bright}HLA-DR⁺ cells remained elevated compared to baseline levels in the CD8 subset ($p = 0.04$). In the CD4 subset, no significant

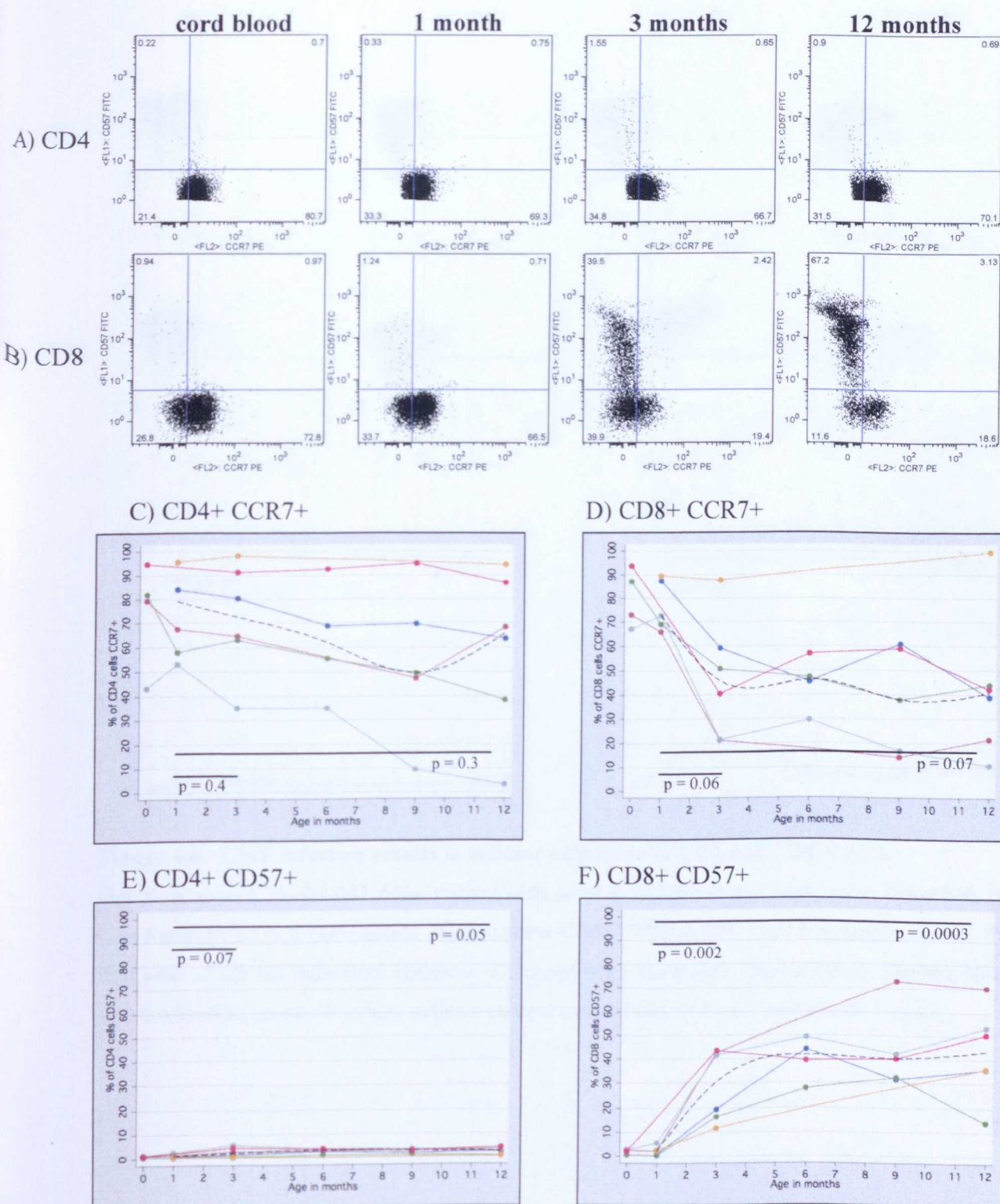


Figure 4.3. CMV infection results in decreased CCR7+ and increased CD57+ frequencies.

Dot plots show CTL-B1-081 cells, stained with panel C antibodies and analysed as described. A) CD4+ and B) CD8 T cell subsets. C&D) CCR7+, E&F) CD57+. Lines show data for individuals and median spline (dashed black line). P values indicate comparisons of month 1 vs 3, and month 1 vs 12.

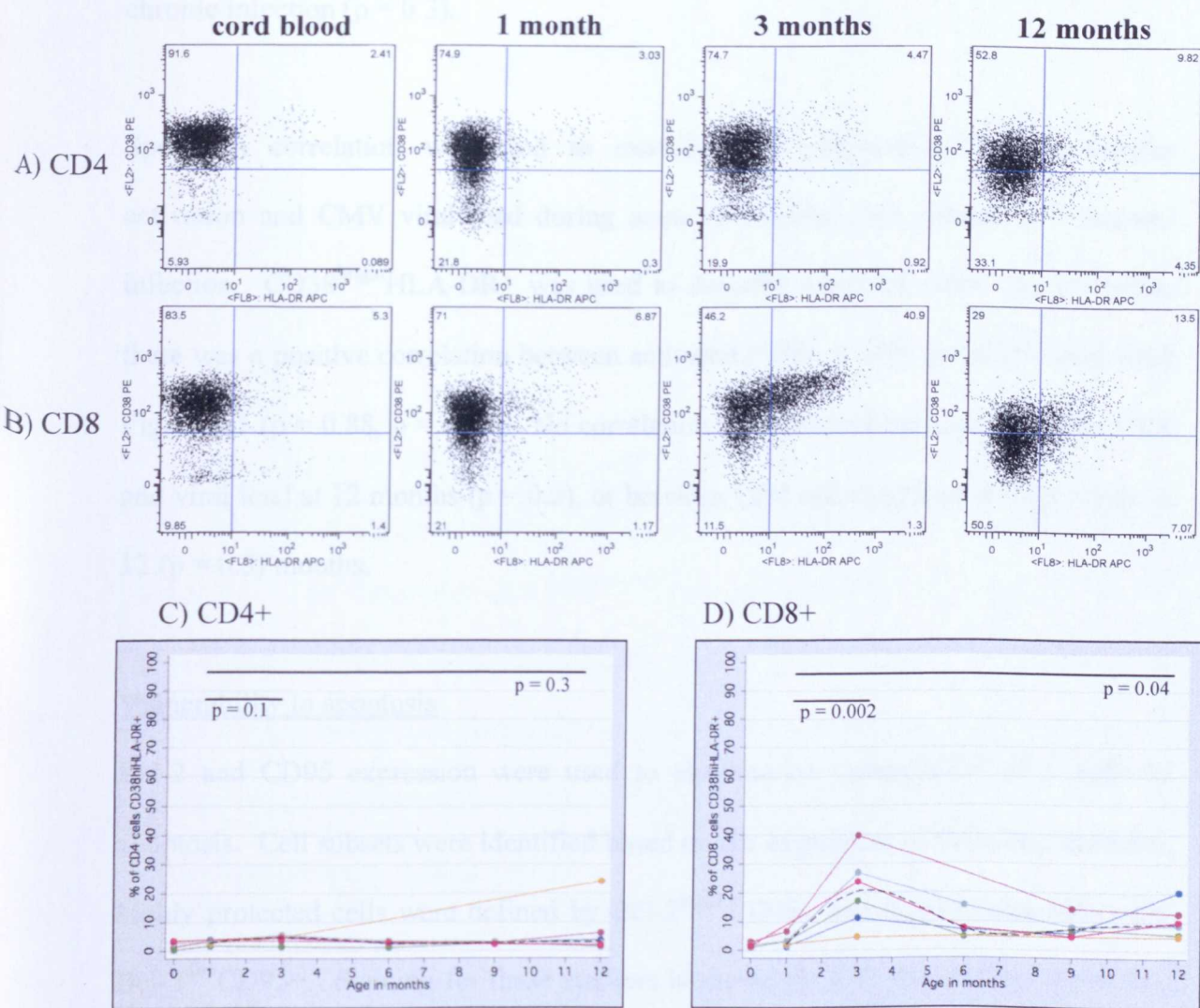


Figure 4.4. CMV infection results in cellular activation of CD4 and CD8 T cells.

Dot plots show CTL-B1-081 cells, stained with panel E antibodies and analysed as described. A) CD4+ and B) CD8 T cell subsets. C&D) show CD38^{bright}HLA-DR+ cell frequencies during the first year of life for individual children. Coloured lines show individual children. Dashed black line is a median spline. P values indicate comparisons of month 1 vs 3, and month 1 vs 12.

In the CD4 subset, many cells expressed CD38^{bright}HLA-DR⁺ at 1 month, but this frequency decreased by 3 months and remained low through 12 months. In the CD8 subset, many cells expressed CD38^{bright}HLA-DR⁺ at 1 month, but this frequency decreased by 3 months and remained low through 12 months. In the CD4 subset, many cells expressed CD38^{bright}HLA-DR⁺ at 1 month, but this frequency decreased by 3 months and remained low through 12 months. In the CD8 subset, many cells expressed CD38^{bright}HLA-DR⁺ at 1 month, but this frequency decreased by 3 months and remained low through 12 months.

changes in CD38^{bright}HLA-DR⁺ were detected from pre-CMV and acute ($p = 0.1$) or chronic infection ($p = 0.3$).

Spearman correlation was used to examine the relationship between cellular activation and CMV viral load during acute (3 months) and chronic (12 months) infection. CD38^{bright}HLA-DR⁺ was used to describe activated cells. At 3 months, there was a positive correlation between activated CD8⁺ T cells and CMV viral load Figure 4.5 ($\rho = 0.88$, $p = 0.02$). No correlation was detected between activated CD8 and viral load at 12 months ($p = 0.2$), or between CD4 and viral load at 3 ($p = 0.6$) or 12 ($p = 0.3$) months.

Vulnerability to apoptosis

Bcl-2 and CD95 expression were used to characterise vulnerability of T cells to apoptosis. Cell subsets were identified based on the expression of these two markers; highly protected cells were defined by Bcl-2^{bright}CD95⁻, and highly vulnerable cells Bcl-2^{dim}CD95⁺. Staining for these markers is shown for CTL-B1-081 in Figure 4.6. Prior to infection, frequencies of vulnerable cells were low. The detection of CMV infection results in the redistribution of cells from protected to vulnerable phenotype. The accumulation of Bcl-2^{bright}CD95⁺ cells was observed primarily in the CD8 subset. In the CD4 subset, many cells expressed CD95, but Bcl-2 was retained during acute CMV infection in the majority of cells. Longitudinal assessment of all the infants showed an increase in vulnerable CD8⁺ cells during acute infection ($p = 0.002$ for 1 vs 3 months) that persisted for at least 9 months post infection ($p = 0.001$ for comparisons at 1 vs 12 months). In the CD4 subset, changes from pre-CMV frequencies were not significant at 3 ($p = 0.4$) or 12 months ($p = 0.06$).

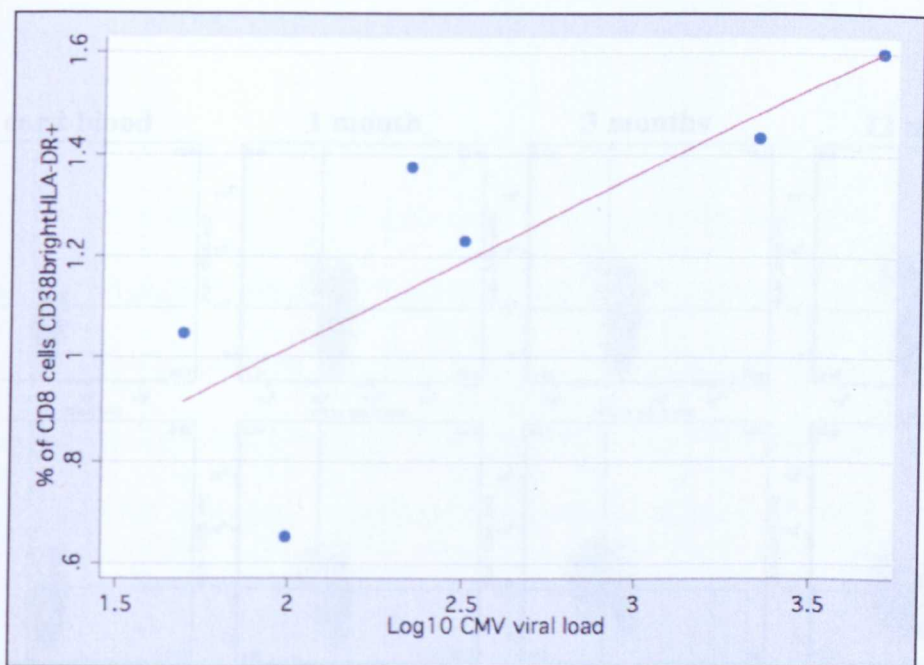


Figure 4.5. The frequency of CD38^{bright}HLA-DR⁺ CD8 T cells correlates with CMV viral load during acute infection.

Scatter plot shows 6 infants with CMV viral load and flow cytometry data collected at 3 months of age. All infants had first CMV detection at 3 months and no detection of HIV-1. Spearman $\rho = 0.88$, $p = 0.02$.

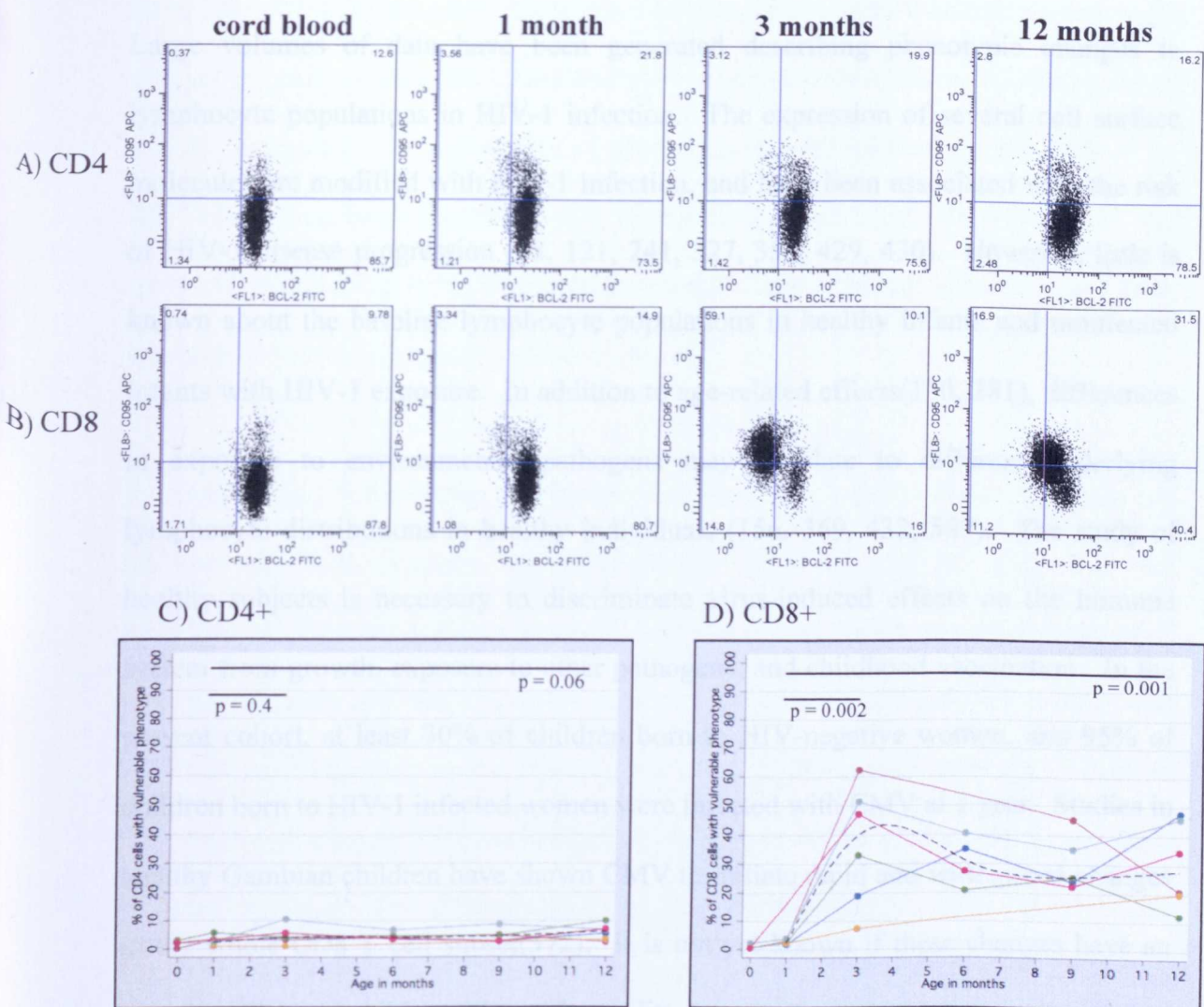


Figure 4.6. Apoptotic vulnerability during CMV infection.

Dot plots show CTL-B1-081 cells, stained with panel E antibodies and analysed as described. A) CD4+ and B) CD8 T cell subsets. C&D) show highly vulnerable Bcl-2^{dim}CD95⁺ cell frequencies during the first year of life. Coloured lines show data for individual children, black dashed line is a median spline. P values indicate comparisons of month 1 vs 3, and month 1 vs 12.

Discussion

Large volumes of data have been generated describing phenotypic changes to lymphocyte populations in HIV-1 infection. The expression of several cell surface molecules are modified with HIV-1 infection, and have been associated with the risk of HIV-1 disease progression (68, 121, 241, 327, 358, 429, 430). However, little is known about the baseline lymphocyte populations in healthy infants and uninfected infants with HIV-1 exposure. In addition to age-related effects(130, 381), differences in exposure to environmental pathogens may translate to different underlying lymphocyte distributions in healthy individuals (156, 369, 432, 587). The study of healthy subjects is necessary to discriminate virus-induced effects on the immune system from growth, exposure to other pathogens, and childhood vaccination. In the present cohort, at least 30% of children born to HIV-negative women, and 95% of children born to HIV-1 infected women were infected with CMV at 1 year. Studies in healthy Gambian children have shown CMV to initiate rapid and widespread changes in the whole CD8 T cell subset(372). It is not yet known if these changes have an adverse effect on child health, or the ability to mount an immune response against other pathogens. Additionally, the study of HIV-1 infected children in this cohort must also take into account the background CMV prevalence and CMV-associated changes as a likely confounder to interpretation of the HIV-1 data. This chapter documents changes in lymphocyte distributions during the first year of life in healthy and HIV-1 exposed uninfected infants, and describes the effect of CMV on T cell subsets.

In the MIP children with no HIV-1 exposure, cord blood cells were primarily naïve, early maturation, apoptosis-protected phenotype. Cellular activation was observed at

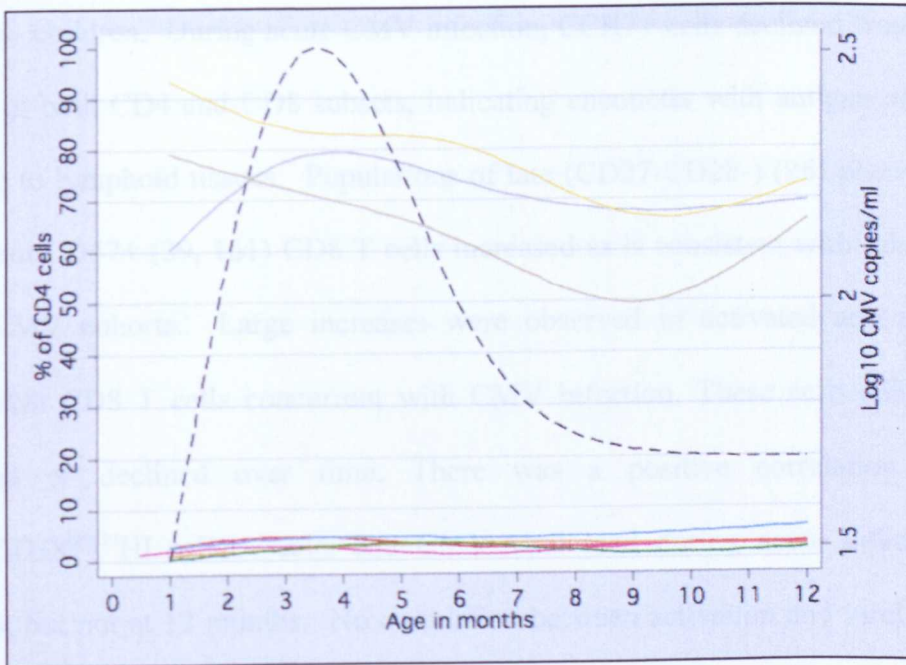
low levels, and low frequencies of proliferating cells were detected. At 6 months, both CD4 and CD8 subsets became more heterogeneous, with evidence of increasing antigen experience, increased populations of apoptotic-vulnerable cells, and increased cellular activation. The appearance of phenotypically differentiating CD8 cells in the MIP infants may be attributable to age, vaccination, or infection. Though at least 3 of these children were infected by CMV 6 months of age it is not possible to say these changes occurred in response to CMV.

A study in the US measured lymphocyte frequencies in a large cohort of infants and adolescents, their markers included CD28, CD45RA, CD38, HLA-DR and CD95(497). The two studies were quite different in their follow-up, the current study was longitudinal and the US study was cross-sectional and grouped infants into intervals of 3 months; so the two studies must be compared with some caution. American and Kenyan children were similarly antigen naïve at birth. However, by 6 months changes in CD4+CD45RA+, CD8+CD45RA+, CD8+CD38+, and CD8+CD95+ cells were observed in the MIP children whilst little difference was seen between the 0-3 and 3-6 month groups in the US study. A longitudinal study by De Vries (123) in the Netherlands also demonstrated similarly naïve cells in cord blood with gradual diversification with age. Studies in Ethiopian children have shown similar phenotypic distributions of memory and activation phenotypes at birth but the development of higher frequencies of activated and memory cells with age (544). In Ethiopia this may be related to persistent helminth infections which activate T cells (269, 273, 274). These studies suggest that environmental factors may have a great impact on lymphocyte subsets during the first few months of life.

There were notable differences between the HIVexp- and unexposed infants, including higher baseline frequencies of Treg (CD4+CD25^{bright}) and apoptotic-vulnerable cells in the HIVexp-. The unexposed infants also had more diverse CD27CD28 subsets at baseline. From this small study it cannot be determined whether these differences are a result of HIV-1 exposure. Though the demographics of the MIP and CTL cohorts are similar, HIV-1 infection in the CTL cohort is likely to mean higher rates of maternal infections from other environmental pathogens (including CMV reactivation, rotavirus, helminths, and tuberculosis), and subsequent infant exposures. It is also not possible to ascertain from this study whether these differences are immunologically significant to the infants, or if there are long-term effects of HIV-1 exposure on cellular immunity. Others have reported differences in CD4 phenotype and function of HIV-1 exposed uninfected children(103), with exposed children having lower naïve cell frequencies and higher frequencies of activated and memory cells. An alternative hypothesis is that exposure to HIV-1 from the mother results in the generation of an adaptive immune response. This is somewhat supported by evidence of HIV-1 specific T cell responses in HIV-1 exposed uninfected infants (299, 300, 316, 506).

Serial measurements of CMV viral load and phenotype in the HIVexp- group enabled the construction of a model of CMV infection involving both the virus and changes in lymphocyte populations. Unfortunately CMV is so prevalent during infancy in Kenya that it was not possible to study CMV-uninfected infants as a control group, and comparisons can only be made with data from other studies. Since the majority of infants became CMV viraemic at 3 months of age, it was possible to standardise the stage of CMV infection relative to the age of the children. Figure 4.7 depicts a

A) CD4



B) CD8

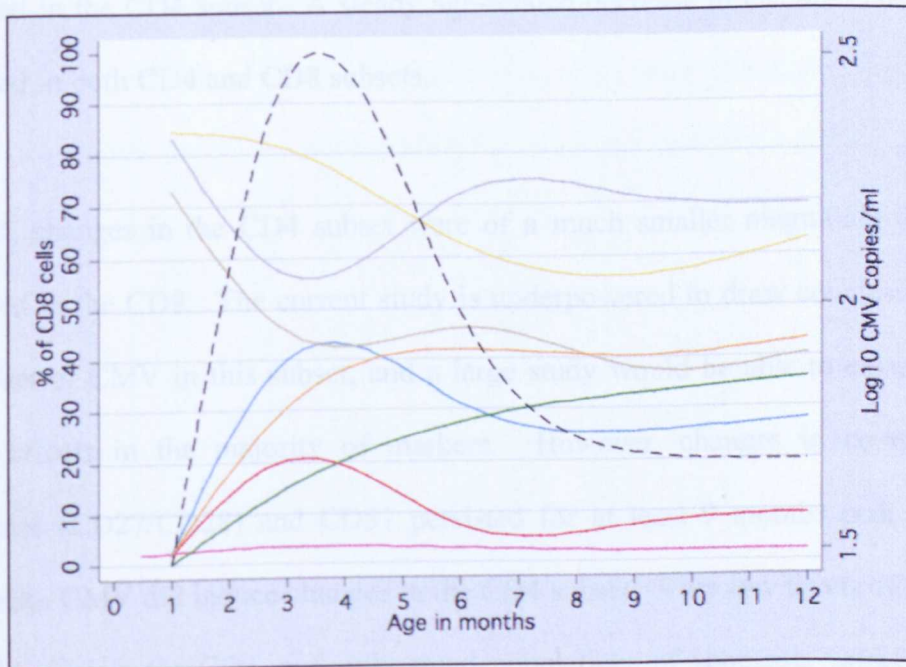


Figure 4.7. Lymphocyte redistributions during CMV infection.

Cubic median splines summarise data for HIVexp- infants to 12 months. All infants became CMV viremic at 3 months. A) CD4 B) CD8. Dashed black = log10 CMV viral load, yellow = CD38^{bright}, gray = CD45RA⁺, brown = naïve (CCR7⁺), blue = apoptotic vulnerable (Bcl-2^{dim}CD95⁺), orange = senescent (CD57⁺), green = late (CD27-CD28-), red = activated (CD38^{bright}HLA-DR⁺), pink = proliferating (CD71⁺)

summary of viraemia and lymphocyte changes during primary CMV infection in HIVexp- children. During acute CMV infection, CCR7+ cells declined from baseline values in both CD4 and CD8 subsets, indicating encounter with antigen and loss of homing to lymphoid tissues. Populations of late (CD27-CD28-) (26) phenotype and senescent CD57+ (39, 161) CD8 T cells increased as is consistent with other reports from CMV cohorts. Large increases were observed in activated and apoptosis-vulnerable CD8 T cells concurrent with CMV infection. These cells subsequently plateaued or declined over time. There was a positive correlation between CD8+CD38^{bright}HLA-DR+ cells and CMV viral load during acute infection at 3 months, but not at 12 months. No correlation between activation and viral load was observed in the CD4 subset. A steady age-related decrease in CD38+ cells was also observed in both CD4 and CD8 subsets.

Overall, changes in the CD4 subset were of a much smaller magnitude than those observed in the CD8. The current study is underpowered to draw conclusions about the effect of CMV in this subset, and a large study would be able to examine more subtle effects in the majority of markers. However, changes in co-stimulatory molecules (CD27/CD28) and CD57 persisted for at least 9 months post infection, suggesting CMV did induce changes in the CD4 subset. Very low levels of activation were detected in the CD4, and only small populations of apoptosis-vulnerable cells were observed. The smaller scale of CD4 changes may suggest tighter homeostatic control over activation in this subset. CD4 responses in neonates may be biased toward development of Th2 phenotype(434, 564) and the presence of very high levels of activated cells, especially Th2 cells, could result in levels inflammation dangerous to the host.

Many changes were observed concurrent to the appearance of CMV viraemia in the infants. Though this evidence suggests the changes observed may be CMV-induced, it is not possible to determine from this study what proportion of these changes occur in CMV-specific T cells. The recent paper by Miles and colleagues (372) have demonstrated that changes in the whole CD8 subset largely mirrored what was happening in the CMV-specific CD8 population. A recent study using overlapping peptides to study CD4 and CD8 responses to the entire CMV genome found that CMV-specific cells comprised a very large proportion of memory CD4 and CD8 cells in latently infected (seropositive) people, a median of 9.1% of CD4 and 10.2% of CD8 (526). The size of virus-specific cells during primary infection is likely to be even higher(81, 302, 346, 372, 583). However some of the changes observed here may also be attributed to coincidental infection with other pathogens, for example, Epstein-Barr virus, rotavirus, and helminths are common infections in Kenyan infants.

Summary

Dramatic changes were observed during the first months of life in infants undergoing acute infection with CMV. At birth, CD4 and CD8 T cell subsets were shown to be primarily naïve and to contain low frequencies of activated cells. During acute CMV infection, rapid changes were observed in the CD8 subset that suggested diversification in response to antigenic stimulation, including the appearance of cells with late, activated, apoptotic-vulnerable, and senescent phenotypes. Frequencies of activated CD8 T cells correlated with CMV viral load during acute infection. Changes in the CD4 subset were subtle relative to the CD8 compartment. At 9 months post CMV infection, frequencies of CD4+CD27-CD28-, CD4+CD57+,

CD8+CD27-CD28-, CD8+CD57+, CD8+CD38^{bright}HLA-DR+, and CD8+Bcl-2^{dim}CD95+ cells remained elevated compared to pre-CMV levels.

CHAPTER 5

Lymphocyte redistributions during HIV-1 infection in Kenyan infants

Introduction

Chapter 4 described widespread changes in the distribution of T cell phenotypes in HIV-1 exposed uninfected infants (HIVexp-) during infection with CMV. Many of these same phenotypes are also altered during HIV-1 infection. These include markers of activation (CD38, HLA-DR, CD69, CD95), co-stimulation (CD27 and CD28), proliferation (Ki67, CD71), senescence (CD57), lymphoid homing (CCR7 and CD62L), memory (CD45RA and CD45RO), and apoptosis (Bcl-2 and CD95). Several of these markers have been correlated with increased HIV-1 viral load and disease progression, and it is now widely accepted that persistent immune activation plays an important role in HIV-1 pathogenesis (54, 219, 422).

The study of subjects undergoing acute HIV-1 infection offers insight into the immune-pathogenesis of infant HIV-1 infection. The current chapter describes changes in lymphocyte phenotype during acute and post-acute HIV-1 infection in infants from the CTL cohort undergoing co-infection with CMV.

Materials and methods

Subjects and sampling

A subset of HIV-1 infected (HIV+) children were selected from the CTL cohort based on survival through 3 months of age and specimen availability. Children were chosen if they were HIV-1 RNA or DNA positive by 1 month of age, survived 3 months of follow-up, and had cryopreserved PBMC available for experiments. The HIVexp-children described in Chapter 4 serve as a comparison group of CMV-infected infants for this chapter.

Diagnostic and Virologic assays

HIV-1 diagnosis and viral loads were performed as described in Chapter 2. CMV diagnostics were performed on all infants as described in Chapter 3.

Flow cytometry methods and excluded assays

Cryopreserved PBMC were thawed and stained with antibody panels A-F, and analysis and gating were performed as described in Chapter 4. Due to procedural errors, some infants had incomplete antibody panels: CTL-B1-103 does not have intracellular staining (panels B&D). Upon thawing, the majority of cells from CTL-B1-411 were dead. PBMC from subject CTL-B1-116 were dead at month 9 and PBMC from CTL-B1-161 were dead at month 3 and are excluded from analysis.

Statistical methods

Clinical and laboratory data were collected and stored in a Microsoft Access Database (Microsoft Inc., Redmond, Washington, USA) and analysed using Stata SE v.9 (Stata Corp. College Station, Texas, USA). Cell frequencies and viral loads were \log_{10} -transformed before comparisons. Mann-Whitney rank-sum tests were used to compare median cell frequencies between HIV+ and HIVexp- groups of infants. Correlations were tested using Spearman's rank test. Models of cell frequencies and viral load over time were generated using cubic smoothing splines of time-point medians.

Results

HIV-1 infected children in the study

A summary of follow-up, CD4, viral load, and CMV diagnostic data from the HIV-1 infected (HIV+) children is provided in Table 5.1. As observed in the greater cohort, HIV-1 viral loads were very high and most infants (15/19) were immunosuppressed with CD4% s $< 25\%$. There were 11 deaths among the 19 children (58%) during the two-year observation period, the majority of these were attributed to pneumonia and diarrhoeal illnesses. Two infants were septic at the time of death, one infant had meningitis, and one had tuberculosis.

Phenotype frequencies in HIV-1 infected children

Tables 5.2 and 5.3 show frequencies of selected T cell phenotypes for all HIV+ infants at each study time-point. As observed in the HIVexp- and MIP children, both CD4 and CD8 cells were primarily naïve, protected from apoptosis and resting. Great

changes, marked by increased antigen experience and heterogeneity were observed during the following 24 months in the HIV+ children. These figures are presented for comparison with previous studies measuring lymphocyte subpopulations in paediatric HIV-1 infection. However, by examining all the children together as a group, one cannot know if these effects are induced by HIV-1 or CMV infection.

Infant	HIV detection	Peak HIV VL	^a Set-point HIV VL	Nadir CD4%	Nadir CD4	Month CMV detected	Months of Follow-up	Age at death (months)	Illnesses at death
CTL-045	Birth	2,017,200	168,600	22	2588	3	CBMC-M24		
CTL-093	Birth	5,155,800	2,817,900	20	810	3	CBMC-M24		
CTL-116	Birth	25,313,500	3,976,400	0	6	3	CBMC-M24	24	FTT, pneumonia
CTL-135	Birth	45,033,000	9,011,700	2	129	birth	M1-15	15	FTT, pneumonia, diarrhoea
CTL-156	Birth	312,000	265,700	35	2508	birth	CBMC-M6	6	pneumonia, sepsis
CTL-161	Birth	271,640,000	89,178,000	3	165	1	CBMC-M7	7	FTT, meningitis, TB
CTL-170	Birth	862,400	174,700	17	1414	1	CBMC-M18		
CTL-211	birth	43,129,000	2,978,300	10	617	3	CBMC-M24		
CTL-258	birth	4,775,200	3,872,900	20	925	1	CBMC-M12		
CTL-313	birth	28,073,500	606,900	10	674	3	CBMC-M24		
CTL-040	m1	2,564,600				3	CBMC-M5	5	diarrhoea, sepsis, anaemia
CTL-105	m1	12,213,000				aviraemic	M1-3	4	FTT, pneumonia
CTL-159	m1	8,690,500	614,400	18	621	20	CBMC-M24	24	
CTL-291	m1	17,049,000	7,047,400	9	515	3	CBMC-M15	17	FTT, diarrhoea
CTL-298	m1	31,754,000	1,049,800	12	782	3	CBMC-M7	7	FTT, pneumonia, diarrhoea
CTL-303	m1	41,542,000	3,771,500	17	1639	10	CBMC-M12	13	FTT, pneumonia
CTL-334	m1	2,907,200	1,613,200	24	2372	1	CBMC-M9	11	FTT
CTL-411	m1	14,114,000	3,068,000	15	637	3	CBMC-M24		
CTL-473	m1	22,155,500	13,642,500	6	628	1	M1-18	18	FTT, pneumonia

Table 5.1. Summary of HIV-infected infants patient characteristics.

Illnesses at death indicate major symptoms/disease identified at the time of death, but do not identify a specific cause of death. FTT = failure to thrive, TB = mycobacterium tuberculosis. ^aTwo infants died at the time of their peak HIV-1 viral load and do not thus have set-points defined.

cord blood		1 month	3 months	6 months	9 months	12 months	24 months
(n=15)		(n=14)	(n=15)	(n=13)	(n=10)	(n=8)	(n=4)
Co-stimulation							
Early (CD27+CD28+)		96 (65.7-99.5)	93.65 (82.9-98.1)	89.4 (3.35-98.2)	90.4 (80.4-99.2)	87.3 (27.6-93.9)	92.3 (84.1-100)
Intermediate (CD27-CD28+)		2.17 (0.2-31.2)	3.27 (0.71-13.9)	5.13 (2.15-95.2)	6.38(1.2-13.3)	5.715 (0.86-66.7)	6.04 (1.8-12)
Late (CD27-CD28-)		0.32 (0-7.27)	0.525 (0 .019-5.75)	1.41 (0-20.7)	2.94 (0-5.38)	4.08 (0.042-10.1)	2.24 (0-5.55)
CD45RA+		58.3 (17.1-94.9)	66.1 (49.3-88)	60.2 (9.08-98.7)	58.6 (31.4-99.5)	60.1 (21.1-85.3)	47.8 (38.6-87.4)
^a CD45RA+/early		98 (67.1-99.9)	97.9 (93.6-100)	96.4 (7.77-100)	96.1 (91.7-100)	94.1 (65.4-99.6)	98.1 (93.3-100)
CD45RA+/intermediate		0.83 (0.092-30.4)	0.79 (0-3.05)	1.73 (0-87.4)	1.08 (0.2-2.75)	0.705 (0.27-15.4)	0.925 (0.31-2.3)
CD45RA+/late		0.31 (0-3.79)	0.28 (0-3.11)	0.7 (0-23.1)	0.785 (0-4.71)	1.59 (0-19.2)	0.552 (0-4.24)
Vulnerability to apoptosis							
^a CD45RA-/early		91.6 (57.5-99.3)	85.4 (68.2-96.3)	77.7 (2.23-95.6)	80.3 (44.9-88.8)	77.9 (17.5-87.7)	81.9 (76.3-100)
CD45RA-/intermediate		4.97 (0.31-39)	11.0 (2.11-26.2)	16.2 (1.64-96.7)	14.0 (5.13-18.1)	12.1 (2.05-80.4)	11.9 (3.93-18.2)
CD45RA-/late		0.35 (0-12.1)	1.56 (0.033-14.8)	2.57 (0-32.2)	4.73 (0-46.2)	3.99 (0.087-19.2)	3.31 (0-14.6)
CD152+		0.86 (0.3-3.14)	0.79 (0.27-3.29)	1.26 (0.21-2.16)	0.725 (0.3-2.04)	0.87 (0.17-2.49)	0.535 (0.34-1.58)
Bcl-2bright		91.6 (62.9-97.6)	79.4 (57.5-89.7)	69.7 (25.6-91.7)	75.1 (1.03-89.2)	68.6 (61.2-81.2)	74.2 (55.3-83.3)
CD95+		14.9 (1.86-59.9)	27.0 (3.89-61.6)	28.6 (11.8-82.4)	31 (13.1-49.4)	26.2 (11.1-56.6)	22.1 (11.2-44.2)
Bcl-2brightCD95-		77.4 (37.5-89.1)	60.7 (33.7-84.4)	50.1 (19-75.4)	39.2 (0.6-72.6)	46.9 (27.7-74.9)	51.3 (40.9-69.2)
Bcl-2dimCD95+		1.54 (0.4-4.38)	6.76 (1.02-18.1)	8.35 (4.89-52.3)	13.9 (5.46-47.3)	11.2 (6.05-23.8)	12.2 (5.25-13.7)

Table 5.2. CD4 phenotypes in HIV+ infants (continued on next page). Median cell frequencies (min-max) are shown as percent of CD4 cells, except ^a percent of CD45RA+/- cells. This table includes infants with first HIV-1 infection at birth and 1 month of age.

	cord blood	1 month	3 months	6 months	9 months	12 months	24 months
Homing & proliferation							
CCR7+	91.6 (68.8-98.3)	87.5 (17.4-97.5)	87.3 (70.6-94.4)	77.6 (6.04-94.6)	75.3 (28.1-89.7)	71.1 (24.7-90.5)	72.5 (16.3-84.5)
CD57+	1.32 (0.49-7.66)	2 (0.36-7.68)	2.85 (0.51-5.48)	3.69 (1.69-7.68)	4.16 (1.78-9.87)	3.73 (1.76-18.3)	3.39 (1.27-8.96)
CD71+	2.005 (0.23-4.79)	2.005 (0.11-8.59)	3.7 (0.091-21.5)	4.37 (0.087-26)	5.10 (0.12-14.4)	5.54 (0.038-18.3)	4.04 (0.028-8.1)
Activation							
CD69+	5.22 (0.39-12.6)	2.16 (0.47-14.7)	1.78 (0.43-11.4)	2.51 (0.85-28.2)	1.49 (0.74-12.2)	1.73 (0.19-6.34)	1.25 (1.12-1.33)
HLA-DR+	3.21 (0.58-11.5)	2.88 (1.35-27.4)	6.07 (0.49-13.1)	8.1 (1.89-39.4)	7.17 (1.63-19.3)	4.03 (0.51-48.6)	5.24 (0.39-7.54)
CD38bright	94.4 (76.1-98.2)	90.3 (64.3-97.7)	84.5 (68-94.9)	73.5 (58.5-91)	71.1 (56.2-90.3)	67.1 (31.1-86.8)	77.4 (50.8-86.3)
CD38brightHLA-DR+	2.66 (0.54-9.36)	2.67 (1.3-23.5)	5.15 (0.38-11.8)	6.15 (1.45-33.2)	5.19 (1.16-16.3)	3.34 (0.44-32.4)	3.68 (0.3-5.16)
Treg	6.68 (2.2-18)	4.96 (1.69-10.7)	6.835 (1.33-10.5)	6.59 (0.41-10.9)	6.935 (1.08-11.9)	5.13 (1.51-8.25)	4.17 (3.66-6.04)

Table 5.2. CD4 phenotypes in HIV+ infants

Median cell frequencies (min-max) are shown as percent of CD4 cells. This table includes infants with first HIV-1 infection at birth and 1 month of age.

cord blood		1 month (n=14)	3 months (n=15)	6 months (n=13)	9 months (n=10)	12 months (n=8)	24 months (n=4)
Co-stimulation							
Early (CD27+CD28+)	79.9 (54.1-95.3)	61.3 (23.6-93)	35.2 (12.7-59.4)	26.3 (2.92-44.3)	17.5 (14.6-56.7)	22.3 (5.98-46)	23.5 (12-47.6)
	16.8 (4.53-31.4)	29.1 (6.89-59.7)	40.3 (30.2-65)	32.8 (28.5-56.1)	30.8 (19.2-57.5)	37.7 (23-44.7)	37.6 (31.5-49.4)
	0.84 (0-27.9)	5.18 (0.027-18.8)	19.8 (1.98-39.1)	40.9 (1.44-62)	50.5 (1.86-64.1)	35.3 (17.1-64.3)	29.3 (19.2-56.4)
	78.3 (45.3-97.7)	58 (23.5-83.9)	42.1 (14.8-67.8)	47.7 (22.6-71.8)	48.3 (32.5-55.5)	42.3 (31.8-56)	39.85 (28.8-56.8)
	83.4 (63.2-96.1)	75.7 (39.2-95.8)	45.5 (13.1-85.4)	32.8 (2.06-62.7)	23.05 (13.5-84.6)	31.6 (1.88-65.8)	39.8 (16.8-62.4)
CD45RA +/intermediate	13.6 (3.62-27.3)	17.3 (4.14-40.1)	27 (15.2-50.9)	23.2 (13.1-46.1)	14.95 (9.83-43)	21.25 (9.27-31.9)	23.0 (19.4-27.7)
	0.17 (0-18.6)	6.18 (0-21.1)	22.7 (0.92-43.3)	50.9 (0.98-76.2)	55.9 (0.86-74.6)	41.2 (16.4-85.4)	35.1 (16.7-61.9)
^a CD45RA-/early		50.9 (11.9-88)	21.1 (12.4-46.9)	18.5 (4.91-37.9)	16.3 (9.93-28)	18 (7.89-38.4)	15.4 (5.59-34.8)
CD45RA-/intermediate	31.7 (8-53)	42.95 (11-73)	55.3 (39.4-74.4)	47.3 (33.1-81.9)	43.7 (23.4-71.5)	47.1 (35.1-55.4)	47.5 (44.6-58.2)
	2.64 (0-47.6)	4.79 (0.13-19.8)	14.3 (3.08-38.6)	34.7 (1.87-51.2)	42.4 (1.7-59.8)	33.0 (16.2-54.5)	27.3 (20.1-49.3)
CD152+	1.18 (0.34-18.9)	0.65 (0.15-2.78)	1.42 (0.052-6.77)	0.86 (0.056-8.37)	1.64 (0.097-6.58)	4.04 (0.21-9.47)	2.25 (1.73-6.0)
Vulnerability to apoptosis							
Bcl-2bright	84.6 (58.9-97.3)	58.8 (24.7-90.1)	31.7 (7.04-80.7)	23.1 (4.29-67)	19.1 (0.33-69.5)	33.4 (18.9-49.6)	42.5 (37.3-62.1)
	18.2 (7.01-63)	42.2 (25.6-78.8)	60.2 (29.6-88.4)	63.7 (37.1-84.5)	69.6 (40.2-88)	56.6 (47.3-76.6)	55.9 (50.6-91.3)
Bcl-2brightCD95-	72.7 (33.3-89.8)	33.9 (11.6-69.6)	19.9 (2.66-43.3)	18.1 (1.3-47.1)	12 (0.15-49.4)	20 (9.78-36.7)	27.2 (5.96-39.4)
	9.63 (0.5-30.4)	24.4 (2.92-63.5)	47.7 (9.05-82.3)	47.9 (25.6-75.9)	55 (23.5-87.7)	43.3 (28.8-61.3)	41.1 (24.6-55.1)

Table 5.3. CD8 phenotypes in HIV+ infants (continued on next page)

	cord blood	1 month	3 months	6 months	9 months	12 months	24 months
Homing & proliferation							
CCR7+	82.2 (12.7-91.1)	42.6 (6.89-70.8)	28.3 (8.07-58.5)	25.3 (1.06-56.3)	19.7 (4.56-74.9)	11.2 (1.1-18.3)	7.73 (4.23-36.5)
CD57+	6.47 (1.08-26.8)	17 (1.05-41.3)	21.4 (8.9-31.4)	36.1 (20-56.7)	36.7 (11.7-69.9)	37.3 (24.7-58.9)	37.1 (28.9-69.6)
CD71+	2.77 (0.24-13.3)	8.39 (3.42-13.5)	6.23 (3.59-16)	5.31 (2.33-20.6)	3.23 (1.37-12.2)	2.72 (1.42-10.8)	3.01 (1.34-4.46)
Activation							
CD69+	7.38 (1.52-17.6)	6.56 (1.36-45.3)	6.28 (1.62-41.4)	6.9 (2.4-26.8)	7.57 (4.5-14.4)	5.42 (1.38-21.9)	3.37 (2.85-8.21)
HLA-DR+	5.27 (0.64-21.4)	24.9 (3.92-59.7)	48 (5.05-73)	32 (3.62-48.7)	25.8 (3.12-60.9)	25.1 (1.21-57.4)	14.9 (1.77-19.4)
CD38bright	88.3 (60.5-99.2)	91.6 (67.2-98.4)	92 (65.3-98.6)	87 (50.7-98.4)	85.0 (72.3-99.1)	76.1 (31.9-91.6)	80.7 (44.4-92.1)
CD38brightHLA-DR+	5.19 (0.61-21.4)	22.9 (4.15-58.6)	46.6 (5.24-71.4)	28.5 (3.72-47.9)	24.4 (3.34-60.5)	22.8 (1.14-51)	13.8 (1.6-18.5)

Table 5.3. CD8 phenotypes in HIV+ infants

Median cell frequencies (min-max) are shown as percent of CD4 or CD8 cells, except ^apercent of CD45RA⁺/⁻ cells. This table includes infants with first HIV-1 infection at birth and 1 month of age.

HIV-1 induced changes in lymphocyte distributions of infants co-infected with CMV

Longitudinal comparisons were made between HIVexp- and HIV+ groups during the first year of life. CMV infection was detected in 17 of the 18 (94%) HIV-1 infected children with phenotype data. In order to control for timing of CMV infection, comparisons are only made between children with first CMV detection at 3 months of age (8 HIV+ and 6 HIVexp- children). The HIV+ children were analysed as a group because all had HIV-1 infection by 1 month of age. Differences between groups in cord blood and 1 month of age can thus be attributed to HIV-1. The effects of acute CMV infection at 3 months of age were evident in both the CMV+HIVexp- and the co-infected children, and additional changes were observed in the HIV+ group. In general, HIV-1 co-infection resulted in changes remarkably similar to those elicited by CMV, but the magnitude and duration of the lymphocyte redistributions were enhanced by HIV-1.

Co-stimulatory molecules

Changes in CD27 and CD28 expression were observed during acute HIV-1 and CMV infection. Figure 5.1 shows data from several infants with or without HIV-1 or CMV infection. Little change was observed in the CD4 subset in response to either CMV or HIV-1 infection (Figures 5.1A-E). However, both CMV (Figure 5.1G & H) and HIV-1 (Figure 5.1J) infection were accompanied by the redistribution of CD8 T cells from early into more differentiated subpopulations. The infant co-infected at birth had very high frequencies of cells in both the intermediate and late CD8 subpopulations in cord blood (Figure 5.1I).

Figure 5.1. CD27/CD28 phenotype in the CD4 subset during acute infection.

Dot plots show cells gated on lymphocytes/CD3+/CD4+ stained with antibodies from panel A. Rows show A) HIV-unexposed infant with no HIV-1 or CMV B) HIV-unexposed infant CMV first detected at 6 months C) HIVexp- infant CMV first detected at 3 months D) HIV+ infant CMV and HIV detected at birth E) HIV+ infant HIV+ at 1 month CMV+ first detected at 20 months.

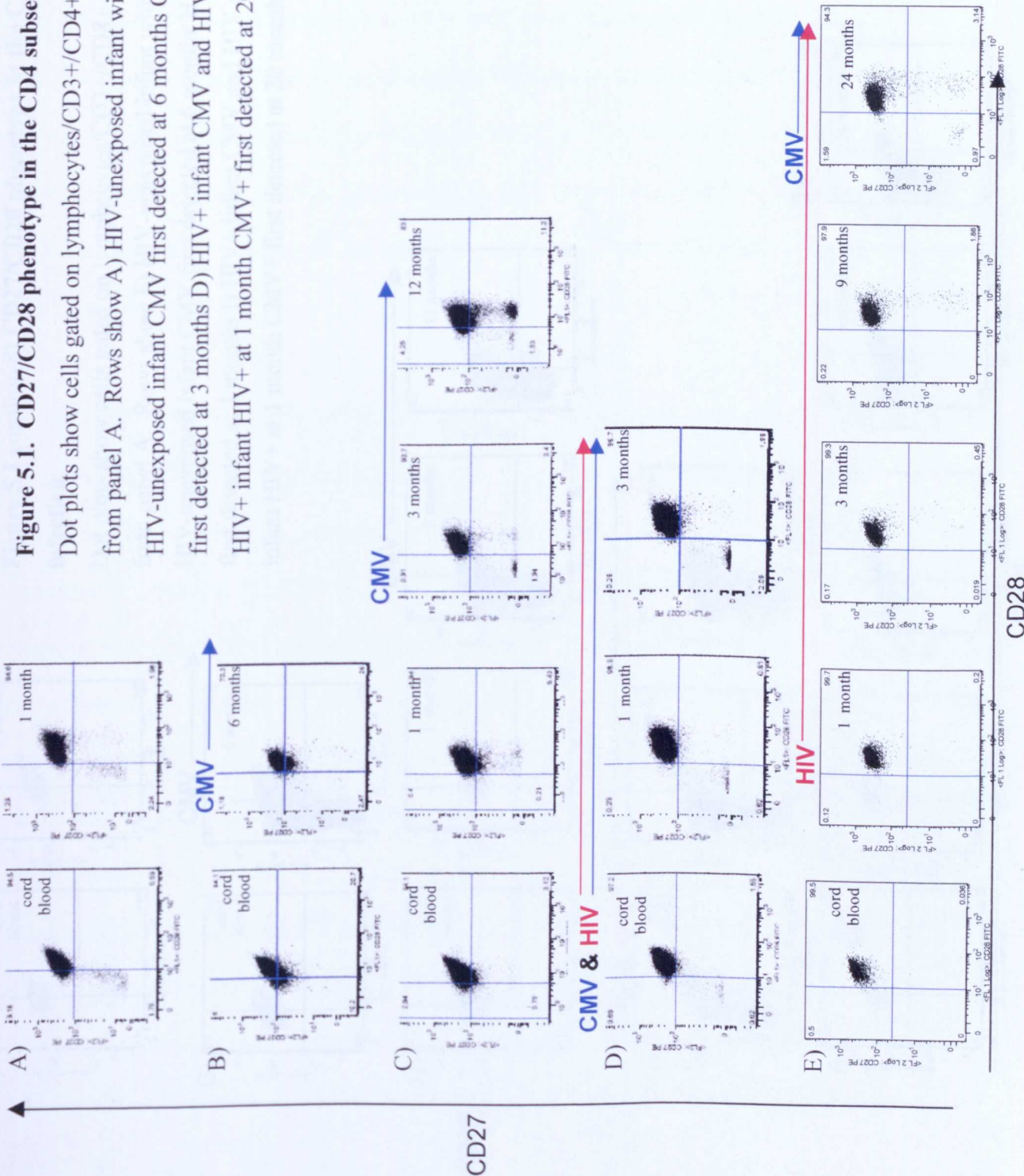
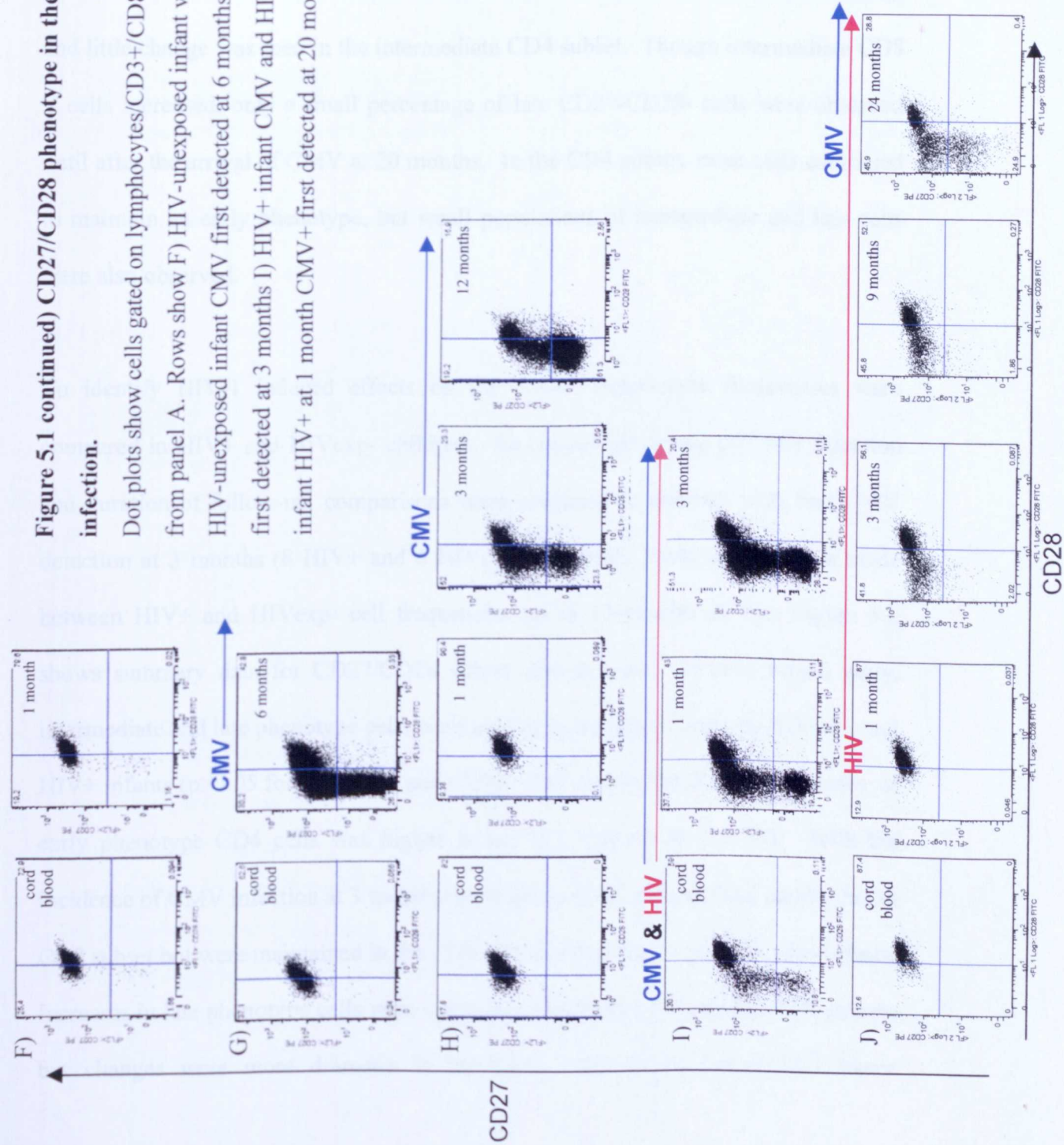


Figure 5.1 continued) CD27/CD28 phenotype in the CD8 subset during acute infection.

Dot plots show cells gated on lymphocytes/CD3+/CD8+ stained with antibodies from panel A. Rows show F) HIV-unexposed infant with no HIV-1 or CMV G) HIV-unexposed infant CMV first detected at 6 months H) HIVexp- infant CMV first detected at 3 months I) HIV+ infant CMV and HIV detected at birth J) HIV+ infant HIV+ at 1 month CMV+ first detected at 20 months.



Patient CTL-B1-159 (Figures 5.1E and J), who was first HIV-1 RNA positive at month 1 and first CMV positive at month 20 is used in the following sections as an example of changes induced by HIV-1. Because HIV-1 and CMV infection were separated by 9 months, this infant's data are especially valuable for visualising the independent effects of the virus on cellular phenotype. Though HIV-1 infection was first detected at 1 month, intermediate CD8 cells were not observed until 3 months, and little change was seen in the intermediate CD4 subset. Though intermediate CD8 T cells increased, only a small percentage of late CD27-CD28- cells were observed until after the arrival of CMV at 20 months. In the CD4 subset, most cells continued to maintain an early phenotype, but small populations of intermediate and late cells were also observed.

To identify HIV-1 induced effects on the cohort lymphocyte frequencies were compared in HIV+ and HIVexp- children. To control for timing of CMV infection and duration of follow-up, comparisons were restricted to children with first CMV detection at 3 months (8 HIV+ and 6 HIVexp- children). Comparisons were made between HIV+ and HIVexp- cell frequencies up to 12 months of age. Figure 5.2 shows summary data for CD27/CD28 subset distributions. In cord blood, early, intermediate and late phenotype cells were similar in frequency between HIVexp- and HIV+ infants ($p > 0.05$ for both CD4 and CD8). At 1 month, the median frequency of early phenotype CD4 cells was higher in the HIV+ group ($p = 0.05$). With the incidence of CMV infection at 3 months, early phenotype cells declined rapidly in the CD8 subset but were maintained in the CD4 cells for both HIV+ and HIVexp- infants. Increases in late phenotype cells were observed in both the CD4 and the CD8 subsets but changes were more dramatic in the CD8. The HIV+ infants had lower

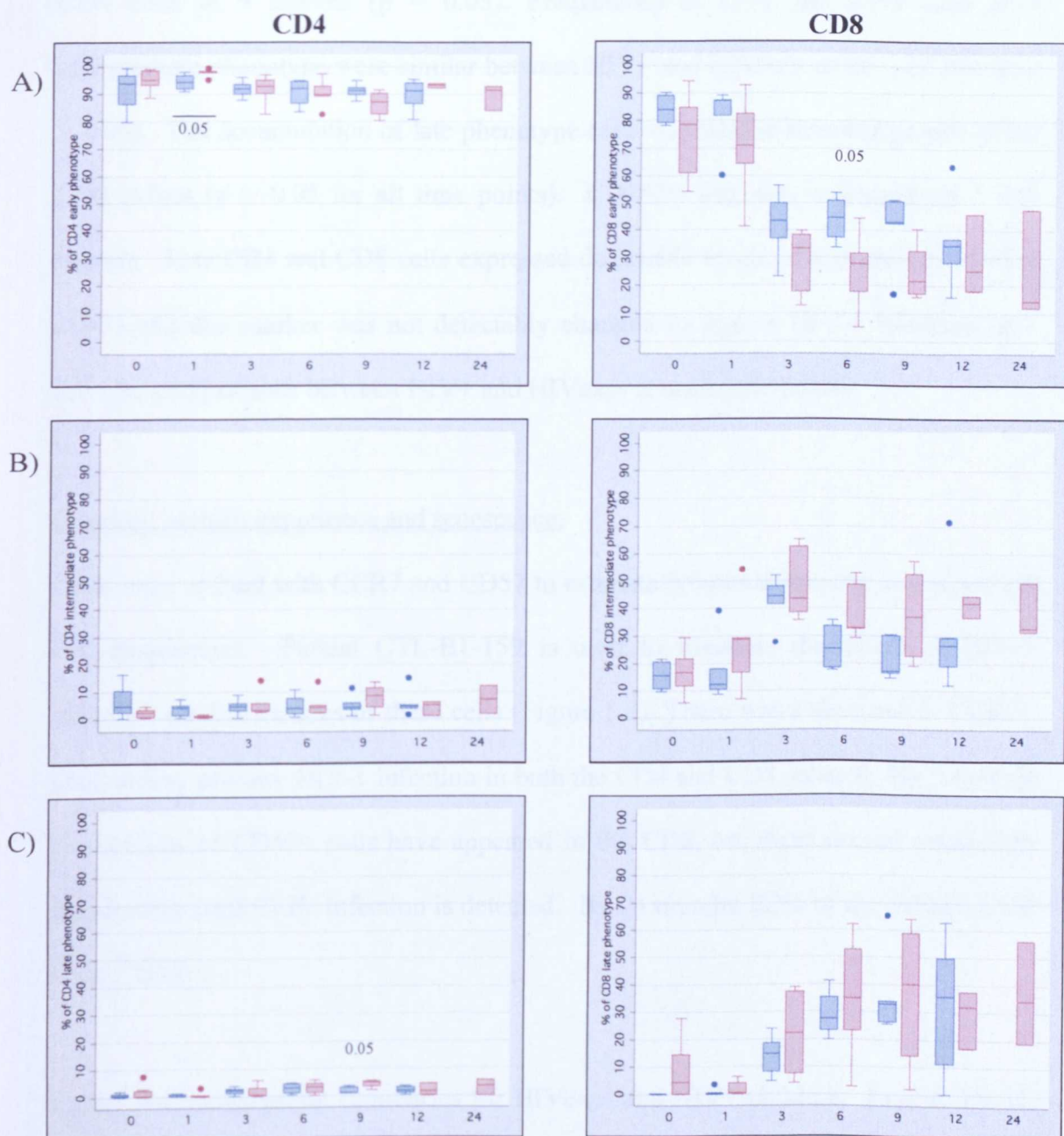


Figure 5.2. Early, intermediate and late phenotype frequencies for HIVexp-CMV+ and HIV+CMV+ infants.

Box plots show frequencies of A) early, B) intermediate and C) late phenotype cells for CD4 (left column) and CD8 (right column) T cells. Red = HIV+ infants, blue = HIVexp infants. Data shown is restricted to children with CMV by 3 months of age. Box plot shows median line, upper and lower box hinges stretch to 25th, and 75th percentiles, whiskers show upper and lower adjacent values, outliers shown as closed circles.

frequencies of early CD8 cells at 6 months ($p = 0.05$) and higher frequencies of late CD4 cells at 9 months ($p = 0.05$). Frequencies of CD4 and CD8 cells with intermediate phenotype were similar between HIV+ and HIVexp- at all time points ($p > 0.05$). The accumulation of late phenotype cells was similar between groups in the CD8 subset ($p > 0.05$ for all time points). CD152+ was also measured on T cell subsets. Few CD4 and CD8 cells expressed detectable levels of this antigen (Tables 5.2-3) and this marker was not detectably changed by age or HIV-1 infection ($p > 0.05$ for comparisons between HIV+ and HIVexp- at each time-point).

Homing, antigen experience and senescence.

Cells were stained with CCR7 and CD57 to estimate lymphoid-homing and senescent cell frequencies. Patient CTL-B1-159 is used to illustrate the effect of HIV-1 infection on distributions of these cells (Figure 5.3). There was a decrease in CCR7+ cells during primary HIV-1 infection in both the CD4 and CD8 subsets. By 3 months populations of CD57+ cells have appeared in the CD8, but these do not reach high frequencies until CMV infection is detected. By 24 months, 22% of the infant's CD8 were CD57+.

Figure 5.4 shows group summaries for HIVexp- and HIV+ children. In cord blood, frequencies of CCR7+, CD57+ and CD45RA+ cells were similar between HIV+ and HIVexp- infants. CCR7+ cells decreased in both the CD4 and CD8 subsets. However in the CD4 subset, HIVexp- infants had lower frequencies of CCR7+ cells compared to HIV+ at 1 month ($p = 0.05$). CCR7+ cells declined more rapidly in the CD8 subset, and in this subset HIV+ infants had a lower frequency of CCR7+ cells at 1 month ($p = 0.05$). The accumulation of senescent (CD57+) cells was observed

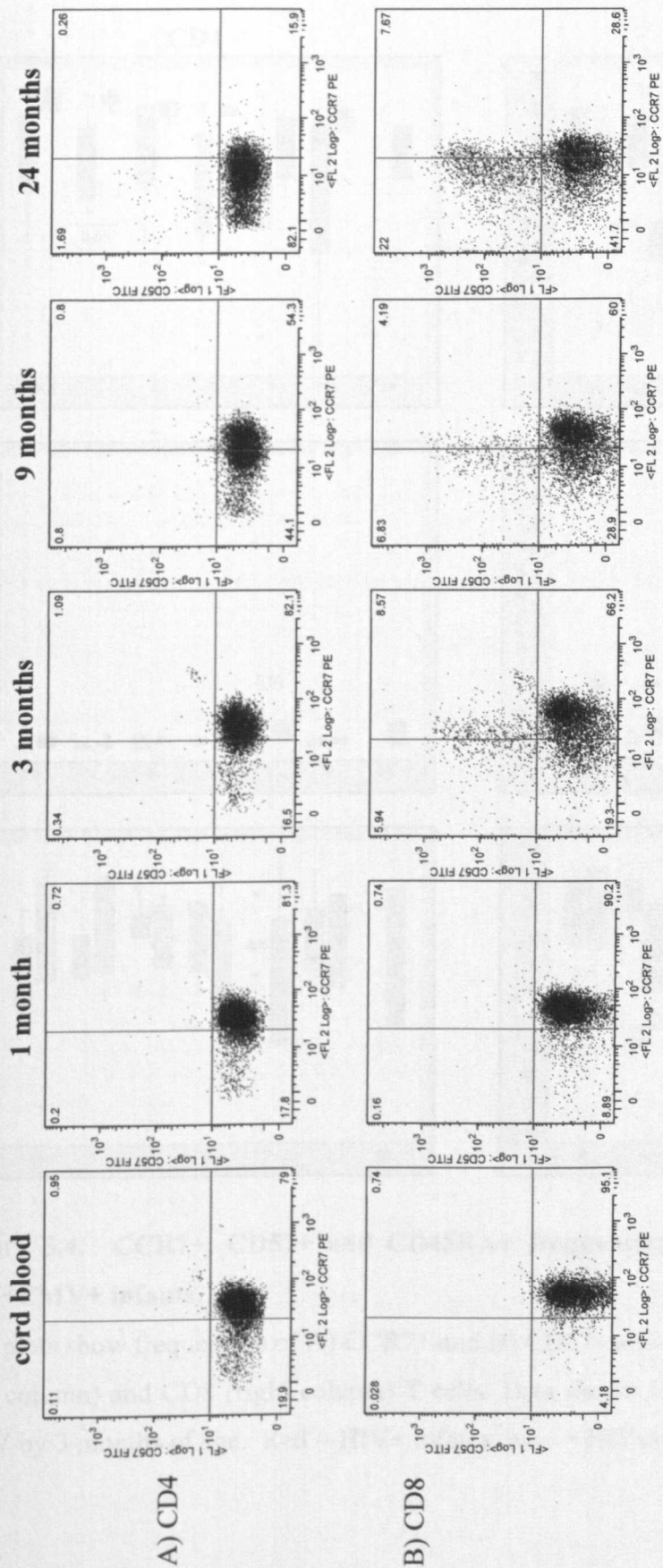


Figure 5.3. HIV-1 and CMV initiate differential changes in CCR7 and CD57 T cell phenotype.

Dot plots show cells from HIV+ infant CTL-B1-159 in cord blood, 1, 3, 9 and 24 months. Cells were stained with antibody panel C, 5000 events are shown for A) CD4+ and B) CD8 T cell subsets. HIV-1 RNA was first detected in the infant at 1 month of age. CMV DNA was first detected at 20 months of age.

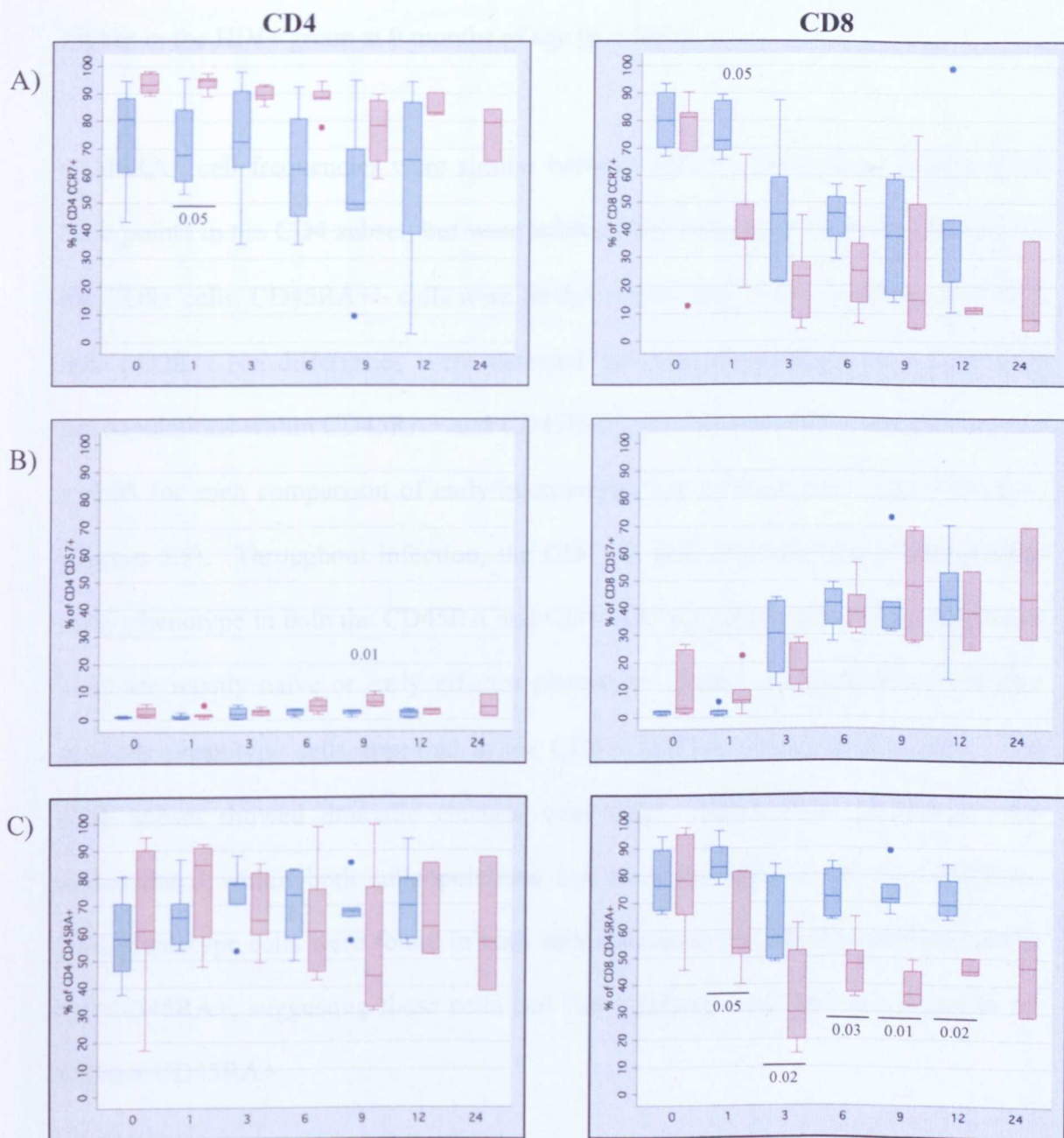


Figure 5.4. CCR7+, CD57+ and CD45RA+ frequencies for HIVexp-CMV+ and HIV+CMV+ infants.

Box plots show frequencies of A) CCR7+ and B) CD57+ and C) CD45RA+ cells for CD4 (left column) and CD8 (right column) T cells. Data shown is restricted to children with CMV by 3 months of age. Red = HIV+ infants, blue = HIVexp- infants.

primarily in the CD8 subset and was not detectably increased in the children co-infected with HIV-1 ($p > 0.05$ for each time-point). CD4+CD57+ frequencies were higher in the HIV+ group at 9 months of age ($p = 0.01$).

CD45RA+ cell frequencies were similar between HIV+ and HIVexp- infants at all time points in the CD4 subset, but were substantially reduced throughout infection in the CD8+ cells. CD45RA+/- cells were further subdivided by the expression of CD27 and CD28. No differences were detected between distributions of CD27CD28 subpopulations within CD45RA+ and CD45RA- gates between HIV+ and HIVexp- ($p > 0.05$ for each comparison of early/intermediate/late between HIV+ and HIVexp-) (Figure 5.5). Throughout infection, the CD4+ T cells maintained a predominantly early phenotype in both the CD45RA and CD45RA- subpopulations, suggesting these cells are mostly naïve or early effector phenotype. Small populations intermediate and late phenotype cells appeared in the CD4+CD45RA- subset at 3 months. The CD8 subset showed dramatic changes over time. Intermediate phenotype cells accumulated within both subpopulations but were predominant in the CD45RA-. Late phenotype cells were found in both subpopulations and became predominant in the CD45RA+, suggesting these cells had fully differentiated and had begun to re-express CD45RA+.

Transferrin receptor CD71 was used as a surrogate marker to quantify proliferating cells. Though dynamic changes were observed in CCR7 and CD45RA during HIV-1 infection, they were not accompanied by increases in proliferation as measured by CD71. CD71 remained constant during the period of observation, and was not significantly different between groups of HIV+ and HIVexp- infants (Figure 5.6). No

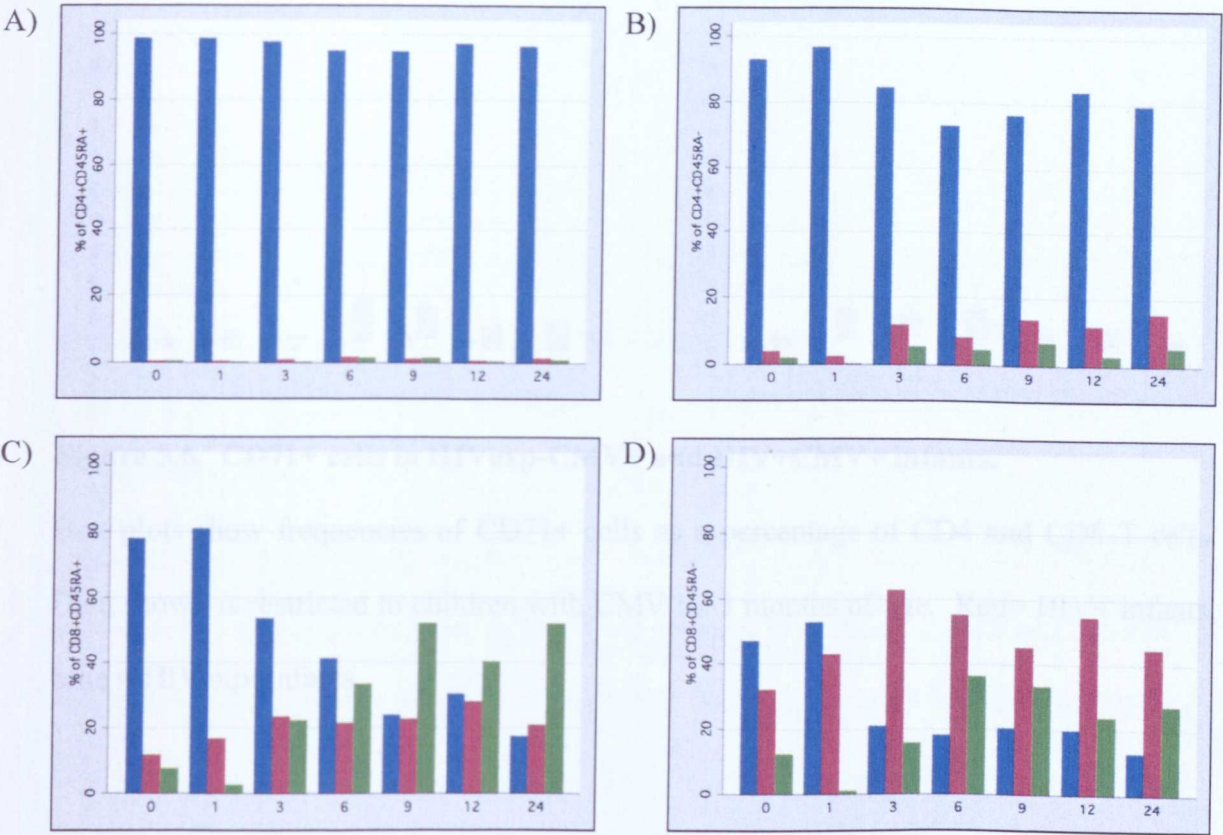


Figure 5.5. Distribution of phenotypic subsets within CD45RA+/- subpopulations in HIV-1 infected infants.

Bar graphs show median group frequencies of early, intermediate, and late phenotype cells in HIV-1 infected infants grouped by CD45RA expression. A) CD4+CD45RA+ B) CD4+CD45RA- C) CD8+CD45RA+ D) CD8+CD45RA-. Blue = early, red = intermediate, green = late phenotype cells.

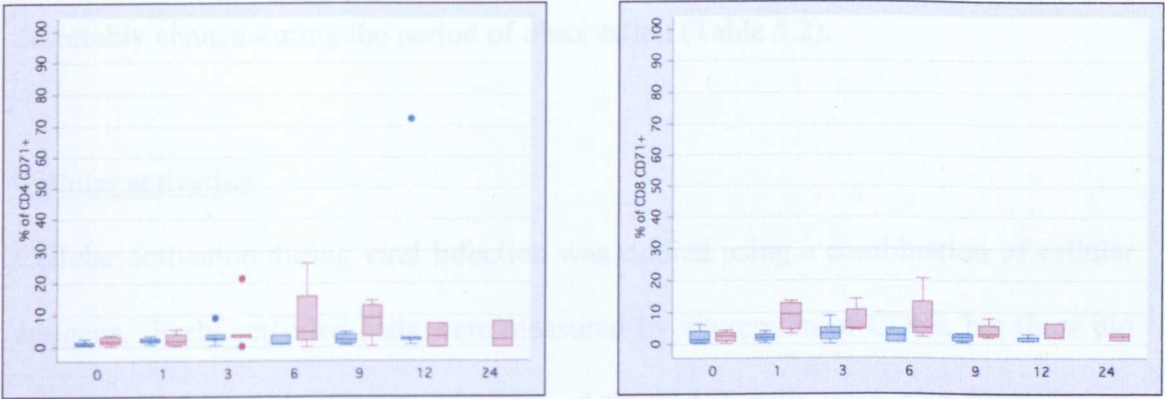


Figure 5.6. CD71+ cells in HIVexp-CMV+ and HIV+CMV+ infants.

Box plots show frequencies of CD71+ cells as a percentage of CD4 and CD8 T cells. Data shown is restricted to children with CMV by 3 months of age. Red= HIV+ infants, blue = HIVexp- infants.

differences were detected in frequencies of Treg (CD4+CD25^{bright}) cells between the HIV+ and HIVexp- infants at any point during observation ($p > 0.05$) and did not detectably change during the period of observation (Table 5.2).

Cellular activation

Cellular activation during viral infection was studied using a combination of cellular antigens. Early activated cells were measured by expression of CD69, but these did not change detectably during infection. Activated T cells were also identified as CD38^{bright}HLA-DR+. Figure 5.7 shows cellular activation in CTL-B1-159. Cellular activation was observed by expansion of CD38^{bright}HLA-DR+ cells in the CD8 subset at 1 month, these cells persisted through follow-up in the infant. Activated CD4 were present from birth in the infant (16%) and their numbers declined by 1 month (5.6%). At 3 months activated CD4 increased to 21%, but then frequencies declined and remained at a lower level even following CMV infection at 20 months (6.8%)

Comparison of HIVexp- and HIV+ infants revealed higher frequencies of CD38^{bright}HLA-DR+ CD8+ cells in HIV+ infants at 1 and 3 months of age ($p = 0.02$, $p = 0.04$, respectively, Figure 5.8). Low levels of cellular activation were observed in the CD4 subset, but no difference was detected between the HIVexp- and HIV+ groups. CD69 expression was expressed at low levels in HIV+ and HIVexp- infants and no significant expansions were observed concurrent HIV-1 or CMV infection.

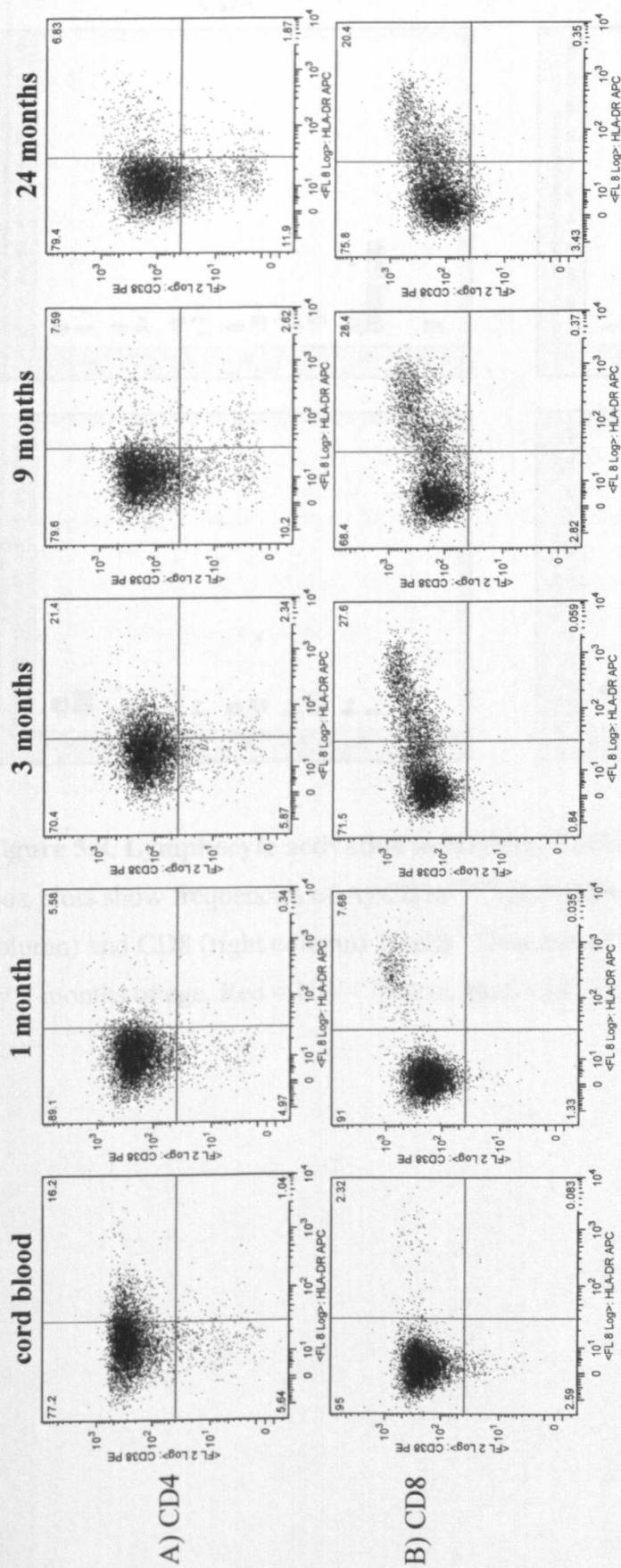


Figure 5.7. Lymphocyte activation during HIV-1 and CMV infection.

Dot plots show cells from HIV+ infant CTL-B1-159 in cord blood, 1, 3, 9 and 24 months. Cells were stained with antibody panel E, 5000 events are shown for A) CD4+ and B) CD8 T cell subsets. HIV-1 RNA was first detected in the infant at 1 month of age. CMV DNA was first detected at 20 months of age.

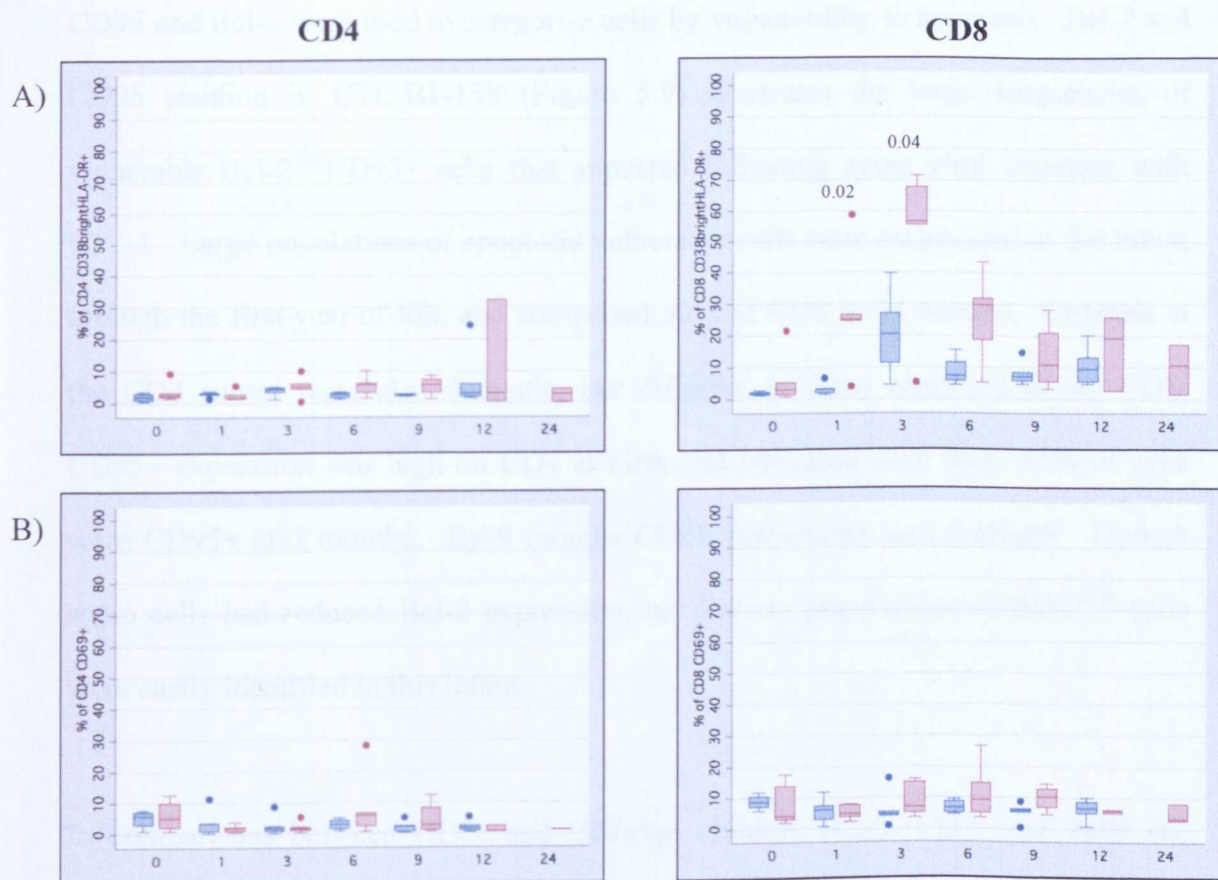


Figure 5.8. Lymphocyte activation in HIVexp-CMV+ and HIV+CMV+ infants.

Box plots show frequencies of A) CD38^{bright}HLA-DR+ and B) CD69+ cells for CD4 (left column) and CD8 (right column) T cells. Data shown is restricted to children with CMV by 3 months of age. Red = HIV+ infants, blue = HIVexp- infants.

Vulnerability to apoptosis

CD95 and Bcl-2 were used to categorise cells by vulnerability to apoptosis. Bcl-2 and CD95 staining in CTL-B1-159 (Figure 5.9) illustrates the large frequencies of vulnerable Bcl-2^{dim}CD95⁺ cells that appeared following acute viral infection with HIV-1. Large populations of apoptotic vulnerable cells were maintained in the infant through the first year of life, and comprised 50% of CD8 at 24 months. Changes in the CD4 subset were also dramatic, but different to those observed in the CD8. CD95⁺ expression was high on CD4 at birth and increased such that ~85% of cells were CD95⁺ at 3 months. By 9 months CD95 frequencies had declined. Though some cells had reduced Bcl-2 expression, no discrete populations of Bcl-2^{dim} cells were easily identified in this infant.

In comparisons between HIV⁺ and HIVexp⁻ children Bcl-2, CD95 and cells co-expressing both markers were examined. In comparisons between HIV⁺ and HIVexp⁻ children an increase in vulnerable cell frequencies was observed in both CD4 and CD8 subsets with increasing age (Figure 5.10). In the CD4 subset, HIV⁺ infants had higher frequencies of vulnerable cells at 3 ($p = 0.04$) and 9 ($p = 0.03$) months of age. Changes in the CD8 were more pronounced, HIV⁺ children had higher frequencies of vulnerable cells at 1 ($p = 0.01$) and 3 ($p = 0.05$) months of age. In the CD8 subset, HIV⁺ infants had higher frequencies of CD95⁺ cells in cord blood ($p = 0.03$) and at 1 ($p = 0.01$), 3 ($p = 0.03$), 6 ($p = 0.01$), 9 ($p = 0.03$), and 12 ($p = 0.04$) months. CD8+Bcl-2^{bright} cells were lower in HIV⁺ children compared to the HIVexp⁻ at 1 month ($p = 0.01$).

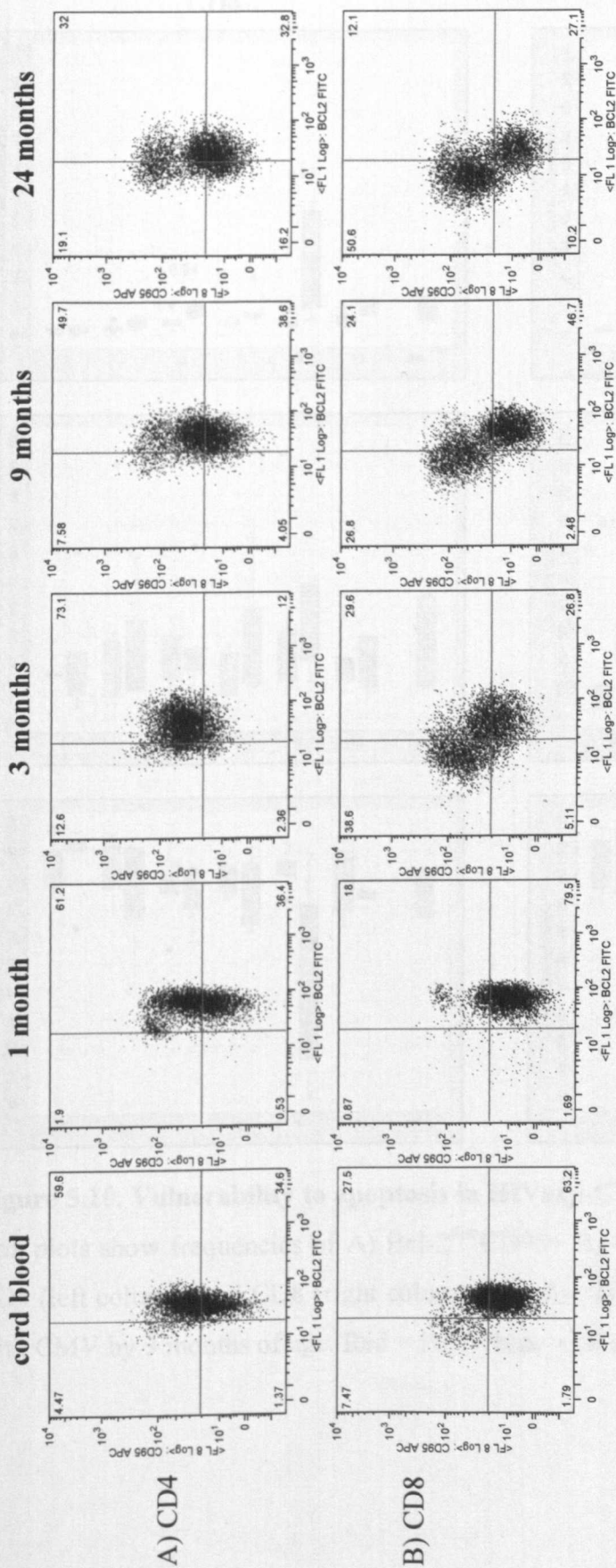


Figure 5.9. Vulnerability to apoptosis in HIV-1 and CMV infection.

Dot plots show cells from HIV+ infant CTL-B1-159 in cord blood, 1, 3, 9 and 24 months. Cells were stained with antibody panel B, 5000 events are shown for A) CD4+ and B) CD8 T cell subsets. HIV-1 RNA was first detected in the infant at 1 month of age. CMV DNA was first detected at 20 months of age.

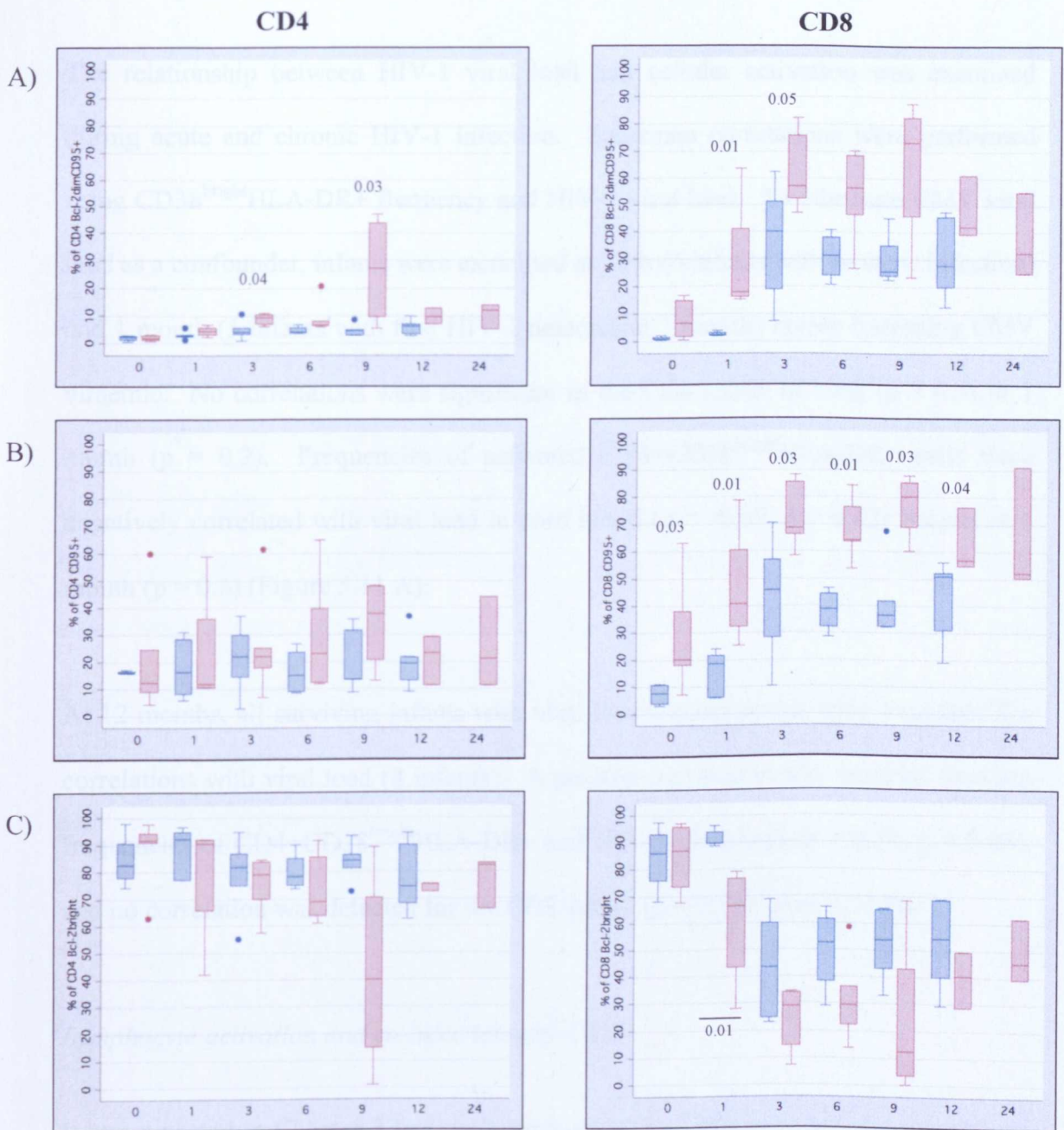


Figure 5.10. Vulnerability to apoptosis in HIVexp-CMV+ and HIV+CMV+ infants. Box plots show frequencies of A) Bcl-2^{dim}CD95⁺ B) CD95⁺ and C) Bcl-2^{bright} cells for CD4 (left column) and CD8 (right column) T cells. Data shown is restricted to children with CMV by 3 months of age. Red = HIV+ blue = HIVexp- infants.

Lymphocyte frequencies and viraemia during HIV-1 infection

The relationship between HIV-1 viral load and cellular activation was examined during acute and chronic HIV-1 infection. Spearman correlations were performed using CD38^{bright}HLA-DR⁺ frequency and HIV-1 viral load. To eliminate CMV viral load as a confounder, infants were examined at birth (6 infants with *in utero* infection) and 1 month (5 infants with first HIV-1 detected at 1 month) before becoming CMV viraemic. No correlations were significant in the CD4 subset at birth ($p = 0.5$) or 1 month ($p = 0.2$). Frequencies of activated CD8+CD38^{bright}HLA-DR⁺ cells were negatively correlated with viral load in cord blood ($\rho = -0.89$, $p = 0.02$) but not at 1 month ($p = 0.6$) (Figure 5.11 A).

At 12 months, all surviving infants with viral load measurements were examined for correlations with viral load (8 infants). A positive correlation was detected between frequencies of CD4+CD38^{bright}HLA-DR⁺ and HIV-1 viral load ($\rho = 0.74$, $p = 0.03$), and no correlation was detected for the CD8 subset ($p = 0.5$) (Figure 5.11 B).

Lymphocyte activation and co-infection with CMV

It was reported in Chapter 3 that early acquisition of CMV infection (by 1 month of age) was associated with increased risk of death in the cohort. To investigate whether co-infection with CMV may increase activation during early HIV-1 infection, frequencies of activated cells were compared between infants at 1 month who had acquired CMV (HIV+CMV+, 6 infants) or had not yet acquired CMV (HIV+CMV-, 8 infants, [Figure 5.12](#)). HIV+CMV+ infants had significantly higher frequencies of CD4+CD38^{bright}HLA-DR⁺ cells at 1 month compared to HIV+CMV- infants (median

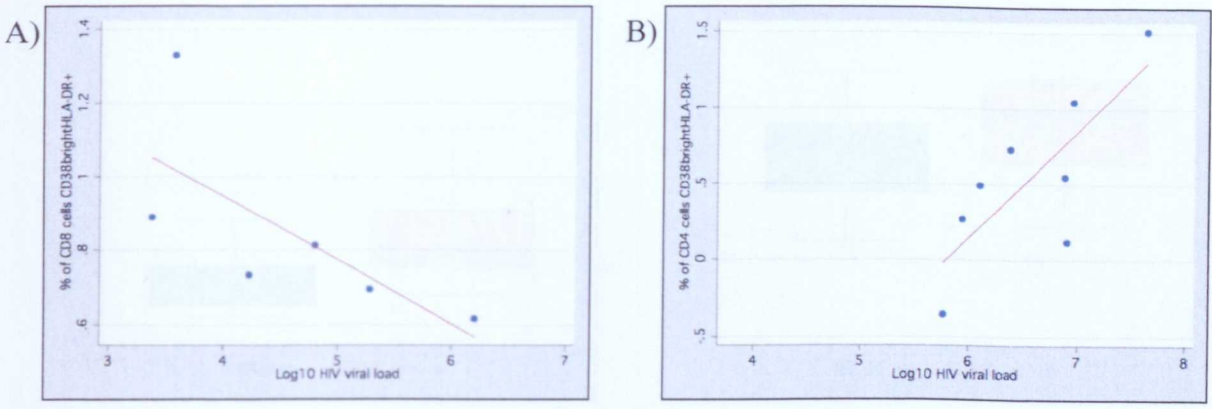


Figure 5.11. Activated cells are correlated with HIV-1 viral load during acute and chronic infection.

A) Frequencies of CD8+CD38^{bright}HLA-DR+ cells are negatively correlated at birth in infants with *in utero* acquisition of HIV-1 (who are CMV aviraemic), $r = 0.89$, $p = 0.02$.

B) At 12 months, frequencies of CD4+CD38^{bright}HLA-DR+ cells are positively correlated with HIV-1 viral load in infants with HIV-1 infection by 1 month of age (*not* adjusted for duration of CMV infection) $\rho = 0.74$, $p = 0.04$.

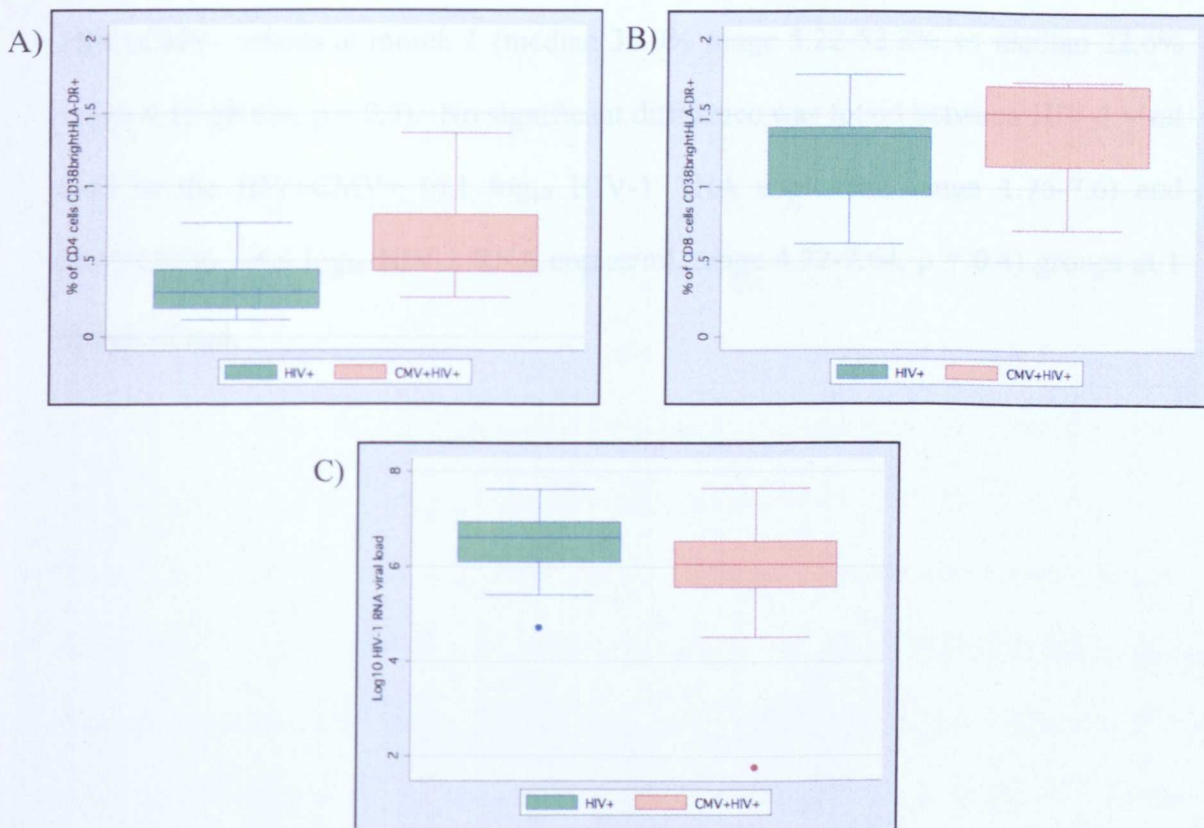


Figure 5.12. CMV co-infection and T cell activation at 1 month of age.

HIV-1 infected infants were analysed at 1 month of age before becoming CMV viraemic. Frequencies of activated A) CD4 T cells are higher at 1 month of age in HIV-1 infected infants with CMV co-infection, $p = 0.05$. B) There is no significant difference between frequencies of activated CD8 between the groups of infants, $p = 0.5$. C) HIV-1 viral load is similar between infants with and without CMV at this time-point $p = 0.4$. Box plot shows median line, upper and lower box hinges stretch to 25th, and 75th percentiles, whiskers show upper and lower adjacent values, outliers shown as closed circles.

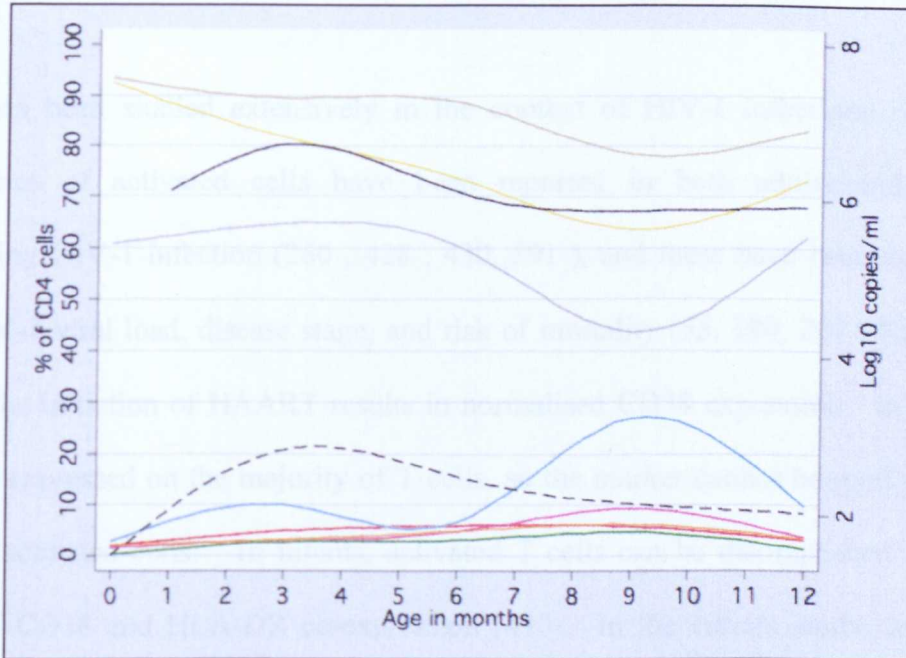
3.27%, range 1.8-23.5% vs 1.98% range 1.3-5.69%, $p = 0.05$). There was no significant difference between frequencies of activated CD8 cells in HIV+CMV+ and HIV+CMV- infants at month 1 (median 35.0% range 5.22-52.8% vs median 22.6% range 4.15-58.6%, $p = 0.5$). No significant difference was found between HIV-1 viral load in the HIV+CMV+ (6.1 \log_{10} HIV-1 RNA copies/ml, range 1.76-7.6) and HIV+CMV- (6.6 \log_{10} HIV-1 RNA copies/ml, range 4.72-7.64, $p = 0.4$) groups at 1 month of age.

Discussion

Dynamic changes were observed in the T cell subsets of the infants undergoing HIV-1 infection. Previous studies examining cellular phenotype in HIV-1 infected infants have been cross-sectional and included children with varying durations of infection. Studies of healthy children have revealed age-related redistributions in lymphocyte populations (156 , 497, 544), so phenotypic data collected from older children may not be comparable to infants. This is also the first report to evaluate CMV as a possible confounder to HIV-1 studies. CMV has been found to have a dramatic effect on T cell phenotype (372), and the very high incidence of CMV in African infants may further confound HIV-1 studies. Ideal controls for these studies would include HIV+CMV- infants and HIV-CMV- infants with similar longitudinal follow-up, but these were not available for the current study. However, by focusing on infants with a constrained timing of HIV-1 and CMV infection, it was possible to somewhat reduce the confounding effect of two infections occurring in a very short space of time. Overall, lymphocyte redistribution in the HIV-1 infected children was very similar to the pattern that was observed during acute CMV infection of HIVexp- infants. Changes were most dramatic in the CD8 though CD4 cells also showed increased heterogeneity suggestive of antigenic stimulation. In the CD8 subset, HIV-1 resulted in increased activation, vulnerability to apoptosis, and the development of memory cells.

A model describing HIV-1 viral load and phenotypes was created analogous to the model of CMV infection ([Figure 5.13](#) and [Figure 4.7](#)). HIV-1 introduces considerable chaos into the distribution of CD8 phenotypes during co-infection with CMV. CMV

A) CD4



B) CD8

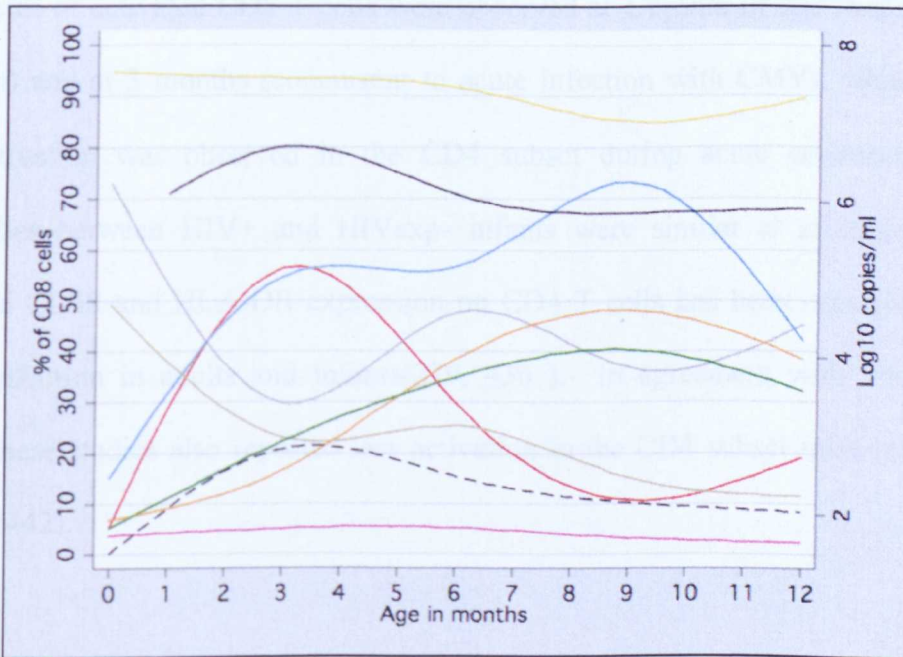


Figure 5.13. Lymphocyte redistributions during HIV-1 and CMV co-infection.

Cubic median splines summarise data for HIV+ infants to 12 months. All infants became CMV viraemic at 3 months and acquired HIV-1 by 1 month of age. A) CD4 B) CD8. Dashed black = log10 CMV viral load, black solid = log10 HIV-1 viral load, yellow = CD38^{bright}, gray = CD45RA+, brown = naïve (CCR7+), blue = apoptotic vulnerable (Bcl-2^{dim}CD95+), orange = senescent (CD57+), green = late (CD27-CD28-), red = activated (CD38^{bright}HLA-DR+), pink = proliferating (CD71+).

and HIV-1 initiated very similar changes in the CD4, with the notable exception of increased frequencies of apoptotic vulnerable cells at 3 and 9 months.

CD38 has been studied extensively in the context of HIV-1 infection. Elevated frequencies of activated cells have been reported in both adults and infants undergoing HIV-1 infection (260 , 428 , 430, 591), and these have been correlated with HIV-1 viral load, disease stage, and risk of mortality (55, 190, 207 , 321, 332 , 499). The initiation of HAART results in normalised CD38 expression. In infants, CD38 is expressed on the majority of T cells, so the marker cannot be used alone to identify activated cells. In infants, activated T cells can be distinguished by high levels of CD38 and HLA-DR co-expression (430). In the current study, increased frequencies of activated CD8 T cells were observed at 1 month of age (before CMV infection) and at 3 months (concurrent to acute infection with CMV). Remarkably little activation was observed in the CD4 subset during acute co-infection and frequencies between HIV+ and HIVexp- infants were similar at all time points. Increased CD38 and HLA-DR expression on CD4 T cells has been reported during HIV-1 infection in adults and infants(279, 430). In agreement with the current cohort, these studies also reported less activation in the CD4 subset relative to CD8 (~10%) (442).

Interestingly, the frequency of activated CD8 T cells was negatively correlated with HIV-1 viral load at birth in infants with *in utero* HIV-1 infection. However, no correlation was observed during acute infection in the infants with first HIV-1 detection of HIV-1 at 1 month. Longer duration of HIV-1 infection may explain why an association was observed at birth and not at 1 month. AZT crosses the placenta

and the suppressive effects of AZT on viral load in the infants infected *in utero* may also contribute to the different trend observed in this group (328). Women in labour were given AZT in accordance with study protocols and this could reduce viraemia in the infants without any participation from the infant immune system (492). Conversely, at 12 months, there was a positive correlation between the frequency activated CD4 cells and viral load, and this is consistent with other reports during chronic infection(279, 430).

CD95 is upregulated on activated T cells, and its expression is associated with increased susceptibility to apoptosis via FasL engagement (17, 73, 133, 267). CD95 and Bcl-2 expression were closely associated in the CD8 subset, with Bcl-2 down-regulation accompanying CD95 expression, and this has been demonstrated to correlate with a high vulnerability to apoptosis (251, 592). However, CD95 and Bcl-2 are indicative of vulnerability to apoptosis, but are not themselves measurements of apoptosing cells. Higher levels of vulnerable (Bcl-2^{dim}CD95+) CD8+ cells were found in the HIV-1 infected children throughout infection, and this is consistent with other reports in adults and children that report increase CD95 or decreased Bcl-2 expression during HIV-1 (30, 64, 358, 420). In the CD4 subset, many cells expressing CD95 continued to express high levels of Bcl-2, suggesting that these cells are not as likely to undergo apoptosis as the CD8 subset. A report by Boudet and colleagues has also shown that CD95 expression on CD4 does not correlate with the down-regulation of Bcl-2(64), though other reports have cited significant decreases in Bcl-2 expression on CD4 from HIV-1 patients (141). The low frequencies of activated and apoptosis vulnerable CD4 cells detected in the blood may be poorly reflective of the true scale of CD4 apoptosis, which primarily occurs in the tissues.

The majority of CD4 depletion has been shown to occur in the gut-associated-lymphoid tissue (GALT), and though peripheral CD4 counts recover following acute infection or ART in adults, the depletion in the GALT persists(69, 220, 325, 362).

CCR7 is lost upon acquisition of effector phenotype, enabling cells to move to the periphery and home to areas of inflammation. CCR7⁺ and early phenotype (CD27⁺CD28⁺) cells were persistently high in the CD4 subset during infection. In the CD4 subset, substantial frequencies of CD45RA⁻ cells were detected, although these were not significantly different from the HIVexp⁻ group. This is in contrast to the CD8 subset, which had markedly reduced frequencies of CCR7⁺ and early phenotype cells over time. Changes in CD45RA cell frequencies were also more dramatic in the CD8 subset, and these changes were amplified by the presence of HIV-1. HIV-1 infection did not significantly alter the distribution of early, intermediate, and late phenotypes within the CD45RA^{+/+} subpopulations. In the CD4⁺CD45RA⁺ subpopulation cells were predominantly CD27⁺CD28⁺, suggesting a naïve phenotype(26, 28). In the CD4⁺CD45RA⁻ subset, more differentiation was observed into intermediate and late effector cells, but the subpopulation still consisted of primarily early (CD45RA⁻CD27⁺CD28⁺) phenotype cells at the end of follow-up. CD8⁺CD45RA⁺ cells were a heterogeneous mix of naïve, intermediate, and late differentiated cells with the naïve population steadily declining over time. After 3 months the CD8⁺CD45RA⁻ subset was primarily late and intermediate phenotype. Though these changes suggest differentiation in response to antigen, from these data it is not possible to know what percentage of these changes are occurring in HIV-specific cells. Considering the presence of CMV co-infection, it is likely that

significant populations of CMV-specific CD8 also comprise the CD27-CD28- subsets (26, 39).

Frequencies of senescent CD8+CD57+(68) cells have been reported to increase during HIV-1 infection (313, 409, 436). CD57+ phenotype cells increased dramatically in the CD8 subset of both HIV+ and HIVexp- infants, and an additional HIV-1 induced effect was not observed in this population. A transiently higher frequency of CD4+CD57+ cells was detected in the HIV+ children at 9 months. Though others have reported altered CD69 (121) CD71 (428) and Treg (377, 545) frequencies, no differences were detected between the groups of HIV+ and HIVexp- infants in the current study.

Early infection with CMV was shown in Chapter 3 to be associated with mortality in the HIV-1 infected children. The excess mortality in the children with low HIV-1 set-point viral loads suggests that the effect of CMV may augment HIV-1 disease progression in children that might otherwise have better prognosis. The phenotypic studies have demonstrated increased frequencies of activated CD4 cells during acute HIV-1 in infants with CMV co-infection. The size of the current cohort is too small to study the effect of activation while controlling for viral load, but no difference in HIV-1 or CMV viral load were detected between infants with early and late CMV infection. Together these data suggest that acquisition of CMV during acute HIV-1 infection could potentially affect disease progression by increasing the level of cellular activation.

Summary

Dramatic changes were observed in the CD8 subset in response to HIV-1 infection in the infants. These changes were very similar to those observed during acute CMV infection, but co-infected infants had greater and more sustained frequencies of activated, vulnerable, and differentiating cells. Alternately, changes in CCR7 and CD57 appeared to be primarily attributable to CMV. Changes in the CD4 subset were very small in comparison to the CD8, and this is surprising considering these cells are the primary hosts of HIV-1 infection. Activation of CD4 during acute HIV-1 infection was increased by the presence of CMV co-infection, and this may contribute to the mechanism by which CMV alters survival in HIV-1 infected infants.

Chapter 6

HIV-Specific Immune Responses During Primary

HIV-1 Infection

Introduction

The poor control of HIV-1 infection exhibited by most infants suggests that neonatal T cells are ineffective in limiting viral replication. Much data supports the ability of infants to mount cellular immune responses to viral infection, however responses to different viral infections appear to be quantitatively and qualitatively different. For CMV, the majority of children are able to generate a CMV specific CD8 response to one or more peptides *ex vivo* during early infection (94, 372, 546). Age does not appear to be an impediment to generating strong CMV-specific responses, fully functional effector cells have been detected in during the first weeks of life, and in a foetus at 28 weeks gestation (346). CD8 T cell responses in children with CMV are similar in frequency and effector functions to those described in adults (346) (546) (94). CMV-specific CD8 appear to have diverse effector function, including the production of IFN- γ , MIP-1 β and TNF- α and the capacity for cytotoxicity. The frequency of these cells by tetramer staining also appears to be stable over time, responses measured during early infection are detected many months later at a similar magnitude.

Conversely, the detection of HIV-1 specific CD8 T cells is less frequent in infants and of a smaller magnitude than those observed during early HIV-1 infection in adults

when measured by IFN- γ , IL-2 or cytotoxicity assays (298, 333, 337-339, 359, 487, 489, 506). However, CTL escape mutations are generated by infants suggesting in some cases responses are sufficient to exert selection pressure on virus to mutate (170, 425). Recent studies using overlapping peptides to screen the entire HIV-1 genome have revealed more frequent CD8 responses in infants (534). Infants who survive the first year of life are able to generate stronger responses with increasing age (78, 333, 467). Whether this is due to a survivor effect or a true maturing of the immune response is not known. It has been suggested that high frequencies of regulatory T cells may also limit responsiveness of neonates compared to older children (316, 371).

The mechanisms underlying poorly functional CD8 responses in infant HIV-1 infection are not understood. Sub-optimal priming of T cells or a lack of proper CD4 help may result in the development of antigen-specific CD8 functionally different from adults. HIV-1 specific CD8 T cells from Kenyan infants were examined using tetramer staining and multicolour flow cytometry to determine whether the frequency or phenotype of virus-specific cells was different from adults undergoing acute HIV-1 infection.

Methods

HLA Typing and peptides

DNA was extracted from peripheral blood mononuclear cells with the Puregene DNA Isolation Kit (Puregene, Minneapolis, Minnesota, USA) and used in amplification refractory mutation system-PCR (75) to determine infant HLA types. Peptides were synthesised by Dr. Kati DiGleria using F-MOC chemistry at Weatherall Institute of Molecular Medicine.

ELISpot Assay

ELISpot assays were performed on all infant specimens from the CTL study cohort at 1, 3, 6, 9 and 12 months of age (333). A subset had retrospective ELISpot assays on frozen cord blood specimens after HLA types had been obtained. Peptides were selected on the basis of HLA type from a panel of optimized CTL epitopes previously used for studies of CTL responses in Kenyan sex workers (275) (Table 1). Clade A and D peptide variants were used, as these are the most common subtypes identified in Kenya (393). Since HIV-1 subtyping was not performed as part of this study, epitopes that vary in subtypes A&D were mixed in equal amounts for the ELISpot. Assays were performed on freshly isolated cells obtained from EDTA anticoagulated blood. PBMC were isolated by density gradient centrifugation and resuspended in RPMI medium supplemented with 10% heat inactivated fetal bovine serum (R10). The ELISpot assay was performed as previously described (305) with modifications described in (275). Briefly, 96-well Millipore plates (Millipore Corp, Bedford, UK) were coated with 1-DIK Mab at 15 µg/ml (Mabtech, Nacka, Sweden) and incubated

at 37 °C for 2 h. Plates were washed six times with sterile RPMI then blocked for 30 minutes with R10. PBMC from HIV-1 infected or exposed uninfected subjects were added to the plates in duplicate at 2×10^5 /well and stimulated with either PHA at 20 µg/ml (positive control, Murex Biotech, Dartford, UK), R10 media alone (peptide control), or 20 µM peptide. The plated cells were incubated at 37 °C and 5.0% CO₂ overnight. Following incubation, plates were washed six times with PBS plus 0.05% Tween-20 (Sigma Chemicals Co., St. Louis Missouri, USA) and coated with biotinylated anti-IFN-γ-Mab 7-B6-1 at 1 µg/ml (Mabtech) for 3 h at room temperature. Plates were washed six times with PBS-Tween, followed by the addition of streptavidin-conjugated alkaline phosphatase (1:1000, Mabtech) for 1.5 h at room temperature. Plates were washed again and developed with an alkaline phosphatase-conjugate substrate kit (Bio-Rad Laboratories, Hercules, California, USA) for 10 min or until blue spots were clearly visible in the PHA control wells with the naked eye. The reaction was stopped by washing with tap water. Spots were counted with the AID ELISpot reader and software (Autoimmun Diagnostika, Straßberg, Germany). Spot forming units (SFU) was defined as the average number of spots in duplicate wells, and HIV-1-specific SFU (HIVSFU) was defined as SFU minus the average number of spot forming cells in the negative control wells. A positive response was defined by an assay meeting the following criteria: (1) strong response in the PHA-stimulated positive control wells, (2) HIVSFU at least 50 HIVSFU/10⁶ PBMC after subtraction of no peptide control and (3) SFU at least twice the value of the negative control.

HLA	Protein	Sequence	HLA	Protein	Sequence
A1	P17	GSEELRSLY	B8	Nef	FLKEKGGL
A1	Rev	ISERILSTY	B8	p24	DIYKRWII/EIYKRWII
A2	RT	ILKEPVHGV/ILKDPVHGV	B8	RT	GPVKQWPL
A2	Nef	ALKHRAYEL	B8	gp160	YLKDQQLL/YLRDQQLL
A2	P17	SLFNTVATL/SLYNTVATL	B8	p17	GGKKKYRL/GGKKKYKL
A2	P24	TLNAWVKVI/TLNAWVKVV	B14/Cw8	p24	DRFFKTLRA/DRFYKTLRA
A2	RT	VIYQYMDDL	B14/Cw8	p24	DLNTMLNTV/DLNMMLNIV
A2	Nef	VLEWRFDSRL	B14/Cw8	p24	RAEQASQEV/RAEQATQEV
A2	Nef	PLTFGWCYKL	B14	gp160	ERYLKDQQL
A3	P17	KIRLRPGGK	B15/Cw4	gp160	SFNCGGEFF
A3	Nef	QVPLRPMTYK	B18/49	p24	FRDYVDRFYK/FRDYVDRFFK
A3	Gp160	TVYYGVVPVWK	B18/49	Nef	YPLTFGWCY/YPLTFGWCF
A3/11/33	RT	AIFQSSMTK/SIFQSSMTK	B27	p24	KRWIILGLNK/KRWIIMGLNK
A3/31	Gp160	RLRDLIIIIVTR	B35	p24	PPIPVGDIY
A3/11/31/33	RT	DLEIGQHRTK	B35	Nef	VPLRPMTY
A3	Nef	DLSHFLKEK	B35	RT	HPDIVIYQY/NPDIVIYQY
A24	Gp160	YLRDQQLL/YLKDQQLL	B35	gp160	TAVPWNASW/TNVPWNSSW
A24/B44	P24	RDYVDRFYKTL/RDYVDRFFKTL	B35	RT	EPIVGAETFY
A24	Nef	DSRLAFHHM	B37/73	Nef	YFPDWQNYT
A25	P24	DTINEEAAEW/ETINEEAAEW	B42	p24	GPGHKARVL
A26/B70/72	P24	YVDRFFKTL	B42	Nef	TPQVPLRPM
A29	Gp160	FNCGGEFFY	B45	RT	GAETFYVDGA
A30	RT	KLNWASQIY	B51	Pol	EPIVGAETFY
A30	P17	RSLYNTVATLY	B53	p24	ASQEVKNWM/ATQEVKNWM
A30	Gp160	IVNRVRQGY	B53	p24	TPQDLNMML/TPQDLNTML
A30	RT	KQNPDIVIYQY	B53	p24	DTINEEAAEW
A30	Gp41	KYCWNLLQY	B53	p24	QATQEVKNW
A6802	Protease	DVTLEDINL	B53	p24	VKNWMTETLL
A6802	Int	ETAYFILKL	B57/5801	p24	TSTLQEQIGW/TSTLQEQIAW
A6802/74	RT	ETFYVDGAAN	B57/5801	p24	KAFSPEVIPMF
A6802/74	Protease	ITLWQRPLV	B57/5801	RT	IVLPEKDSW
A6802	Protease	DTVLEEMNL	B57	p24	ISPRTLNAW/LSPRTLNAW
B7/8101/42	Nef	TPGPGVRYPL/TPGPGIRYPL	B57	Pol	KITTESIWIW
B7	Gp160	IPRRIRQGL	B70/72	RT	DVKQLTEVV/DVKQLAEAV
B7	P24	SPRTLNAWV	Cw4	p24	QASQEVKNW
B7	P24	GPGHKARVL	Cw4	p17	KYRLKHLVW
B7/8101	P24	ATPQDLNTM	Cw8	RT	VTDSQYALGI
B7	Nef	FPVTPQVPLR	Cw8	Gp160	NCSFNISTSI
			Cw8	Nef	KAADVLSMFL

Table 1. Peptides used in IFN- γ ELISpot assays.

Study subjects and selection of specimen subset for tetramer staining

Infants from the CTL study are described in Chapter 2. A subset of infant specimens was evaluated with both ELISpot and tetramer staining. Tetramer staining was not performed on all infants because tetramers are not available for all of the HLA types and epitopes used in this study. Also, experience in the laboratory has shown that tetramer positive cells are rarely identified from specimens with $< 400 \text{ HIVSFU}/10^6$ PBMC in ELISpot assays. Since the production of tetramers is expensive and laborious, only specimens that were previously defined as positive by ELISpot were stained with tetramers. The panel of tetramers available for screening included those previously identified as highly immunodominant in African cohorts that were produced in the Human Immunology Unit: HLA-A03, B08, and B57. Responses to HLA-A29 gp120 were frequently detected in the infants and were very high in magnitude compared to other epitopes. Possession of the HLA-A29 allele was also associated with increased risk of early HIV-1 acquisition in the CTL cohort ($RR = 2.0$)(164).

The algorithm for choosing infants for tetramer staining was: 1) Choose the first specimen with $>300 \text{ HIVSFU}/10^6$ PBMC 2) Choose all available PBMC specimens at study visits prior to and after a specimen meeting criteria (1). Seven infants had ELISpot assay results of sufficient magnitude, and had cryopreserved PBMC available for tetramer staining.

Tetramer staining

In all staining experiments, 3 million cells were resuspended in approximately 200 μ l of R10 media, and tetramer added. The cells were incubated at 5% CO₂, 37°C for 15 minutes. The cells were split into three parts for staining with Panel X, Y, and Z antibodies. Antibodies used in combination with tetramer staining are shown in Tables 2 and 3. Cells not used for tetramers were stained with Panels A-G as described in Chapter 4. Master mixes of antibodies to surface antigens were added to the cells in v-bottom plates and incubated at room temperature in the dark for 20 minutes. The cells were washed twice and fixed with CytoFix (BD Pharmingen, Oxford, UK). Before acquisition, cells were refrigerated at 4°C at least one hour for the inactivation of HIV-1.

For perforin staining, the above protocol was followed for tetramer and surface staining. Instead of the final fixing step, cells were permeabilised by adding 300 μ l of CytoPerm/CytoFix permeabilisation solution (BD Pharmingen) and incubating for 20 minutes in the dark at 4 °C. Cells were washed twice with Perm buffer. Saponin is used in this kit to permeabilise the cells, and since this process is reversible if saponin is removed, cells were kept in the saponin buffer for the remainder of wash and incubation steps. Cells were stained with intracellular antibodies in a volume of 100 μ l for 30 minutes in the dark at 4 °C. Cells were washed twice with Perm buffer and resuspended in 170-200 μ l of Perm buffer.

Fluorochrome	Panel x	Panel y	Panel z
Pacific Blue	CD3	CD3	CD3
PE	Tetramer	Tetramer	Tetramer
PE-Cy5	CD38	HLA-DR	CD38
FITC	CD28	CD57	Perforin
APC	CD27	CD45RA	CD95
APC-Cy7	CD8	CD8	CD8

Table 2. Antibody mixes used in the description of tetramer-stained cells.

Fluorochrome	Antibody	Clone	Manufacturer
PB	CD3	UCHT1	Dako
AC7	CD8	RPA-T8	Pharmingen
APC	CD27	0323	eBioscience
FITC	CD28	CD28.1	Dako
PC5	CD38	HIT2	Pharmingen
APC	CD45RA	HI100	Pharmingen
APC	CD95	DX2	Pharmingen
PC5	HLA-DR	G45-6	Pharmingen
FITC	PERFORIN	dG9	Pharmingen

Table 3. Antibody clones and suppliers.

Analysis and gating

Cells were acquired on a Cyan ADP or LX instrument using Summit software (Dako Cytomation, Angel Drove, UK) as described in Chapter 4. FCS files were analysed with FlowJo software (Treestar, Inc., Olten Switzerland). Data were re-compensated during analysis using single stain controls and FlowJo's automated compensation platform. Cells were first gated by size and granularity, then selected for CD3+ and CD8^{bright} expression. A specimen was classified as "tetramer positive" by the presence of a discrete population of tetramer-stained cells in the CD3+ gated

population what was positive in the CD8^{hi} region, but not the CD8⁻ region. Gates were then placed such that <1% of cells were staining positive in the CD8⁻ quadrant, and then applied to CD3⁺CD8⁺ gated cells to provide tetramer percentages as a proportion of CD8 T cells. Data were exported from FlowJo and uploaded into the FACS LIMS as described in Chapter 4. HIV-1 negative donor cells were used to confirm an absence of staining for each tetramer.

Statistical Analysis

Clinical and laboratory data were collected and stored in a Microsoft Access Database (Microsoft Inc., Redmond, Washington, USA) and analysed using Stata SE v.9 (Stata Corp. College Station, Texas, USA). Cell frequencies and viral loads were log₁₀-transformed before comparisons. Mann-Whitney rank-sum tests were used to compare median cell frequencies between groups of infants. Correlations were tested using Spearman's rank test.

Results

Frequencies of tetramer-stained cells during HIV-1 infection in infants

Data describing overall T cell responses in the CTL study cohort have been published and will not be repeated in detail here (333) (506). ELISpot data were screened for specimens with responses > 300 HIVSFU/10⁶. Responses from this subset were selected if they were directed toward epitopes matching available tetramers. Seven infants met these criteria and had PBMC specimens available for further experiments (Table 4). Four of the infants responded to A29 Env, and 3 infants responded to A3 Nef, B8 Nef or B57 p24.

An example of tetramer staining in and HIV-1 infected infant is shown in Figure 6.1. Very high frequencies were often found during acute infection, HIV-1 specific cell frequencies ranged from 0.14-3.82% of CD8+ T cells. Table 4 summarises frequencies of tetramer+ cells detected in the 7 infants. Correlation analysis revealed a decline in tetramer+ cell frequencies over time (Figure 6.2, $p = 0.03$, $\rho = -0.48$). However, analysis of the whole cohort showed an increase in the magnitude of ELISpot responses over time (333). Tetramer-positive cells were consistently found in specimens that were ELISpot positive above 300 HIVSFU/10⁶. The one exception was CTL-B1-170, at 6 months there was a positive ELISpot response of 685 HIVSFU/10⁶ but no tetramer-positive cells were detected. In some cases tetramer+ cells were detected when IFN- γ responses were measured below 300 HIVSFU/10⁶ (CTL-B1-045 month 6 and CTL-B1-291 month 6). Infant CTL-B1-159 had a negative ELISpot result at 6 months (30 HIVSFU/10⁶ with negative control staining of 25 SFU/10⁶), but a very large population of tetramer-stained cells (2.21%). Otherwise there was good concordance between positive ELISpot responses and the detection of tetramer-positive cells.

Figure 6.3 shows tetramer, ELISpot and HIV-1 viral load data for each infant during two years of observation. Analysis of the entire cohort has previously revealed no relationship between the presence of ELISpot responses or the magnitude of those responses and HIV-1 viral load (333). The relationship between frequencies of HIV-1 specific cells and levels of viraemia may vary depending on the stage of infection (acute or chronic), and the current study is underpowered to rigorously examine the relationship between the two. The most observations were available at 9 months (5 infants) of age, and no significant correlation between HIV-1 viral load and tetramer+

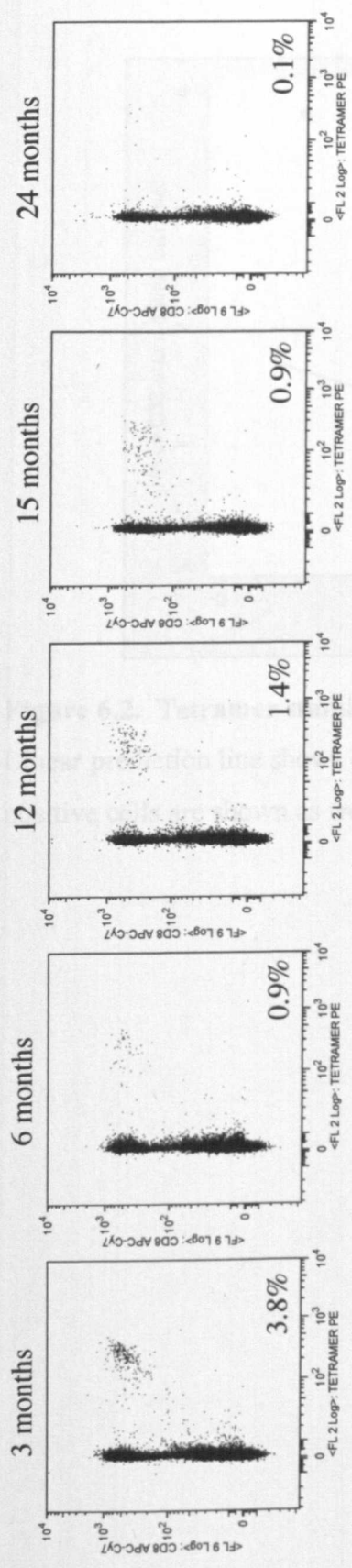


Figure 6.1. HLA-A29 gp120 tetramer staining of CTL-B1-093 from 3-24 months.

Each dot plot shows 30,000 cells from the lymphocytes/CD3+ gate. Percents describe frequency of tetramer positive cells within the lymphocytes/CD3+CD8+ gate.

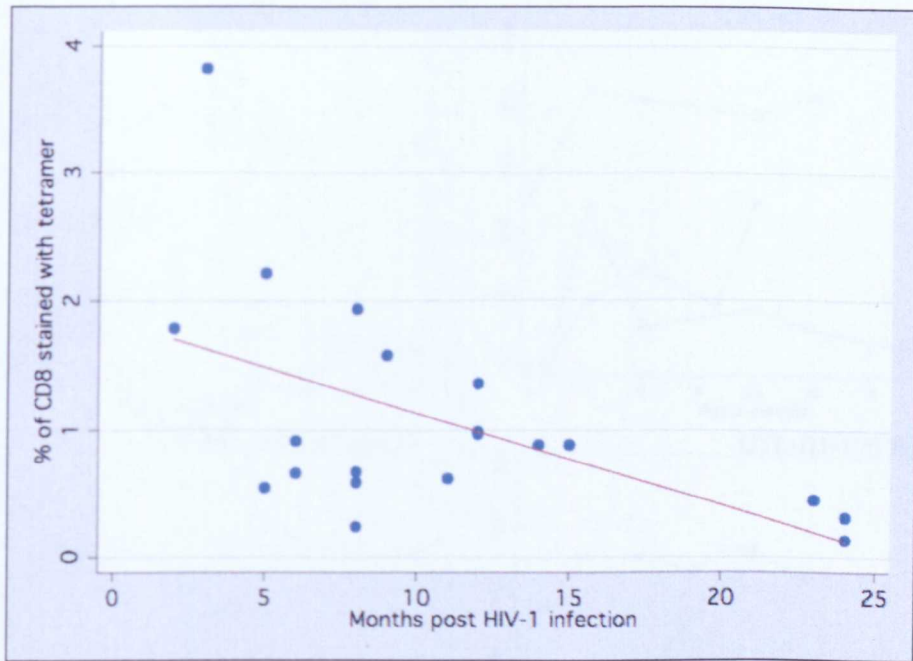


Figure 6.2. Tetramer staining cell frequencies decrease over time.

Linear prediction line shows decrease in tetramer-staining cells over time. Tetramer positive cells are shown as frequency of CD8 T cells. $\rho = -0.48$, $p = 0.03$.

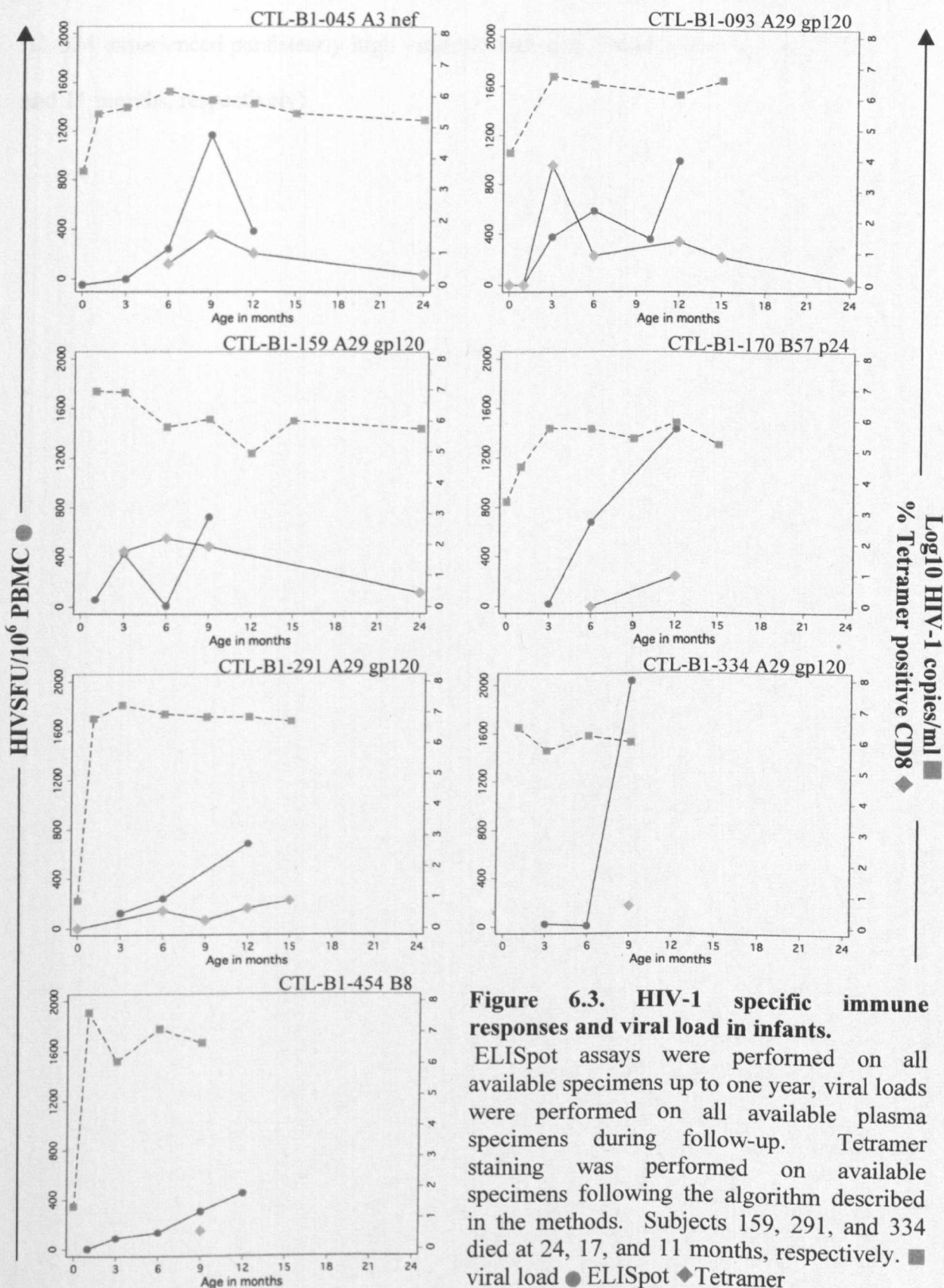


Figure 6.3. HIV-1 specific immune responses and viral load in infants. ELISpot assays were performed on all available specimens up to one year, viral loads were performed on all available plasma specimens during follow-up. Tetramer staining was performed on available specimens following the algorithm described in the methods. Subjects 159, 291, and 334 died at 24, 17, and 11 months, respectively. ■ viral load ● ELISpot ◆ Tetramer

frequency was detected ($p = 0.1$). Despite the generation of high frequencies of tetramer⁺ and IFN- γ -producing cells, subjects CTL-B1-159, CTL-B1-291, and CTL-B1-334 experienced persistently high viraemia and died during follow-up (at 24, 17, and 11 months, respectively).

Subject	HIV+	Class I HLA alleles	Peptide	ELISpot (HIVSFU/10 ⁶)	Tetramer
CTL-B1-045	<i>In utero</i>	A1,A3, B15,B57, Bw4,Bw6, Cw4,Cw7	A3 nef- QVPLRPMTYK	M1: ND M3: negative M6: 248 M9: 1170 M12: 385 M24: ND	M1: ND M3: ND M6: 0.66% M9: 1.58% M12: 0.99% M24: 0.32%
CTL-B1-093	<i>In utero</i>	A29,A74, B14,B1503, Bw6, Cw2	A29 gp120- FNCGGEFFY	Cord: negative M1: negative M3: 373 M6:585 M10:358 M12:993 M15: ND M24: ND	Cord: 0% M1: 0% M3: 3.82% M6: 0.91% M10 :ND M12: 1.36% M15: 0.88% M24:0.14%
CTL-B1-159	<i>Peri partum</i>	A29,A30, B4501, Bw6, Cw6	A29 gp120- FNCGGEFFY	M1: 58 M3: 425 M6: negative M9: 723 M12: ND M24: ND	M1: ND M3: 1.79% M6: 2.21% M9: 1.94% M12: ND M24: 0.45%
CTL-B1-170	<i>In utero</i>	A30,A3402, B42,B57, Bw4,Bw6, Cw7,Cw17	B57 p24- KAFSPEVIPMF	M1: ND M3: negative M6: 685 M9: ND M12:1432	M1: ND M3: ND M6: 0% M9: ND M12:0.96%
CTL-B1-291	<i>Peri partum</i>	A29,A74, B420,B15, Bw6, Cw2,Cw17	A29 gp120- FNCGGEFFY	Cord: ND M1: ND M3: 120 M6: 238 M9: ND M12: 685 M15: ND	Cord: 0% M1: ND M3: ND M6: 0.55% M9: 0.24% M12: 0.62% M15: 0.88%
CTL-B1-334	<i>Peri partum</i>	A29,A26, B13,B15, Bw4,Bw6, Cw2,Cw6	A29 gp120- FNCGGEFFY	M1: ND M3: negative M6: negative M9: 2040 M12: ND	M1: ND M3: ND M6: ND M9: 0.67% M12: ND
CTL-B1-454	<i>Peri partum</i>	A2,A31, B8,B15, Bw6, Cw7,Cw8	B8 nef- FLKEKGGL	M1: negative M3: 90 M6: 135 M9: 303 M12: 450	M1: ND M3: ND M6: ND M9: 0.59% M12: ND

Table 4. ELISpot and tetramer staining in HIV+ infants of the CTL cohort.

M = age of infant in months. ND = not tested at time-point (infant missed clinic visit or no PBMC specimen available). ELISpot results are shown as HIVSFU/10⁶ PBMC, tetramer as % of CD8^{bright}. For a full description of ELISpot responses in the cohort, please refer to (333).

Phenotype of tetramer positive cells

The phenotype of tetramer positive cells was described by staining with antibody panels X, Y, and Z. Phenotype data was not available for infant CTL-B1-454, or infant CTL-B1-291 at months 12 and 15. CTL-B1-170 did not have perforin staining performed. Due to the small sample size of this study, it was not practical to make comparisons between phenotypes for T cells directed against different HIV-1 epitopes, so all HIV-1 tetramer data was analysed as one group. HIV-1 specific cells had a phenotype distinct from the general CD8 population (Figure 6.4). The percentages of HIV-1 specific cells distributed among the major phenotypes at different ages are shown in Table 5.

As previously described in adults, the majority of HIV-1 specific cells in all infants were of the intermediate (CD27+CD28-) co-stimulatory phenotype, however a significant proportion of the tetramer+ cells were also early (CD27+CD28+) and late (CD27-CD28-). The tetramer positive fraction remained predominantly intermediate, > 40% of cells were intermediate in each infant throughout infection (Figure 6.5).

HIV-1 specific cells were highly activated (CD38^{bright}, HLA-DR+, CD95+). Serial observations in four infants showed the majority of tetramer+ cells expressed activation markers on the cell surface throughout infection, though the density of HLA-DR+ and CD38^{bright} expression on cells declined over time (Figure 6.6)

In the majority of infants, HIV-1 specific cells were predominantly CD45RA- throughout the observation period, suggesting an effector or memory phenotype. CTL-B1-045 was an exception, whose A3 cells were predominantly CD45RA+, suggesting a terminally differentiated memory phenotype (Figure 6.7). CD57+

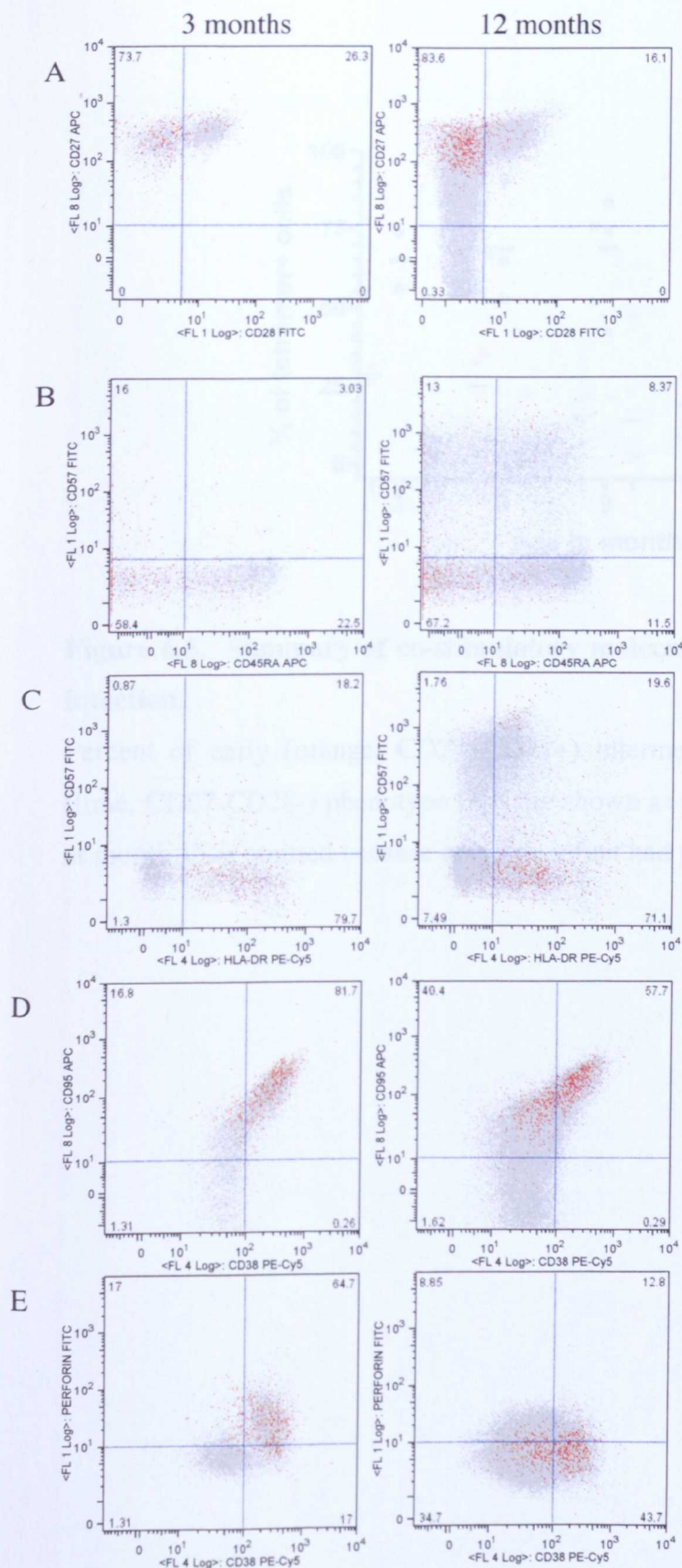


Figure 6.4. HIV-1 specific CD8 T cells have a phenotype distinct from the greater CD8 subset.

Representative staining from infant CTL-B1-093 at 3 and 12 months of age. HIV-1 specific cells (gated: lymphocytes /CD3+CD8^{bright}/ tetramer+) are shown in red, overlaid on the whole CD8 subset (gated: lymphocytes/ CD3+CD8^{bright}) shown in grey. HIV-1 specific CD8 were predominantly intermediate in phenotype (CD27+CD28-(A)). Most HIV-1 specific cells were CD45RA-, and CD57+ cells accumulated with time (B). Compared to the whole CD8 subset, HIV-1 specific CD8 were highly activated, expressing both HLA-DR (C) and CD38^{bright} (D). HIV-1 specific cells expressed CD95 (D). Perforin was found in the majority of HIV-1 specific cells during acute infection, but levels decreased over time (E).

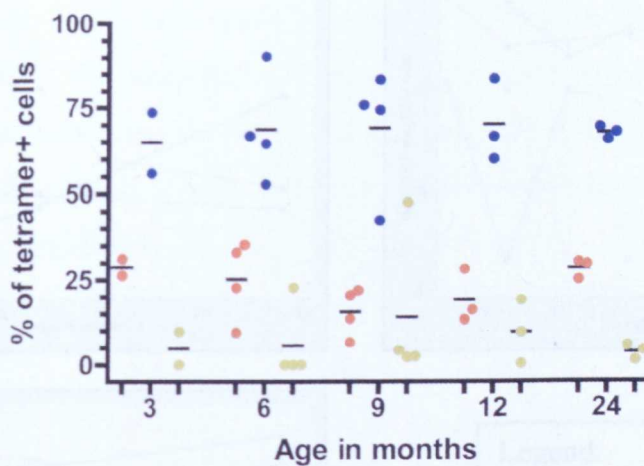


Figure 6.5. Summary of co-stimulatory molecule expression in infants with HIV-1 infection.

Percent of early (orange, CD27+CD28+) intermediate (blue, CD27+CD28-) and late (lime, CD27-CD28-) phenotype cells are shown as percent of tetramer-gated cells. Data at month 15 is omitted because only one infant had phenotype data at this age.

Figure 9.A. Expression of activation markers on HIV-1 specific CD8 T cells.

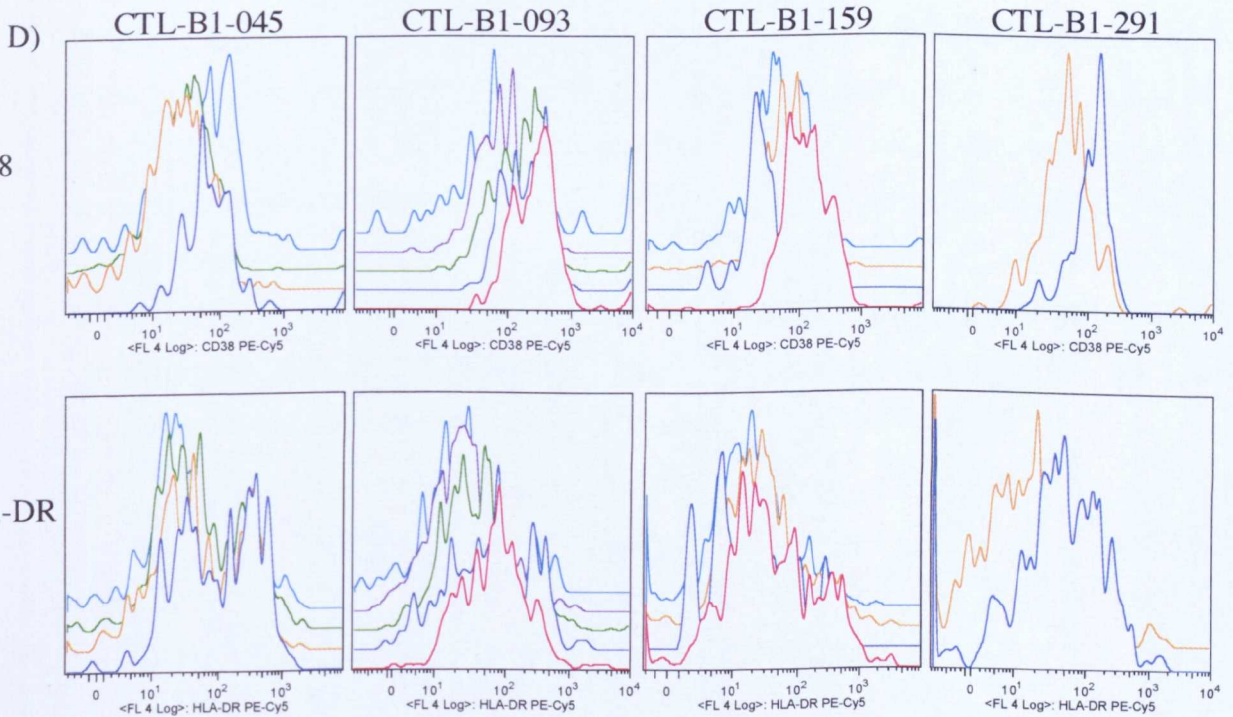
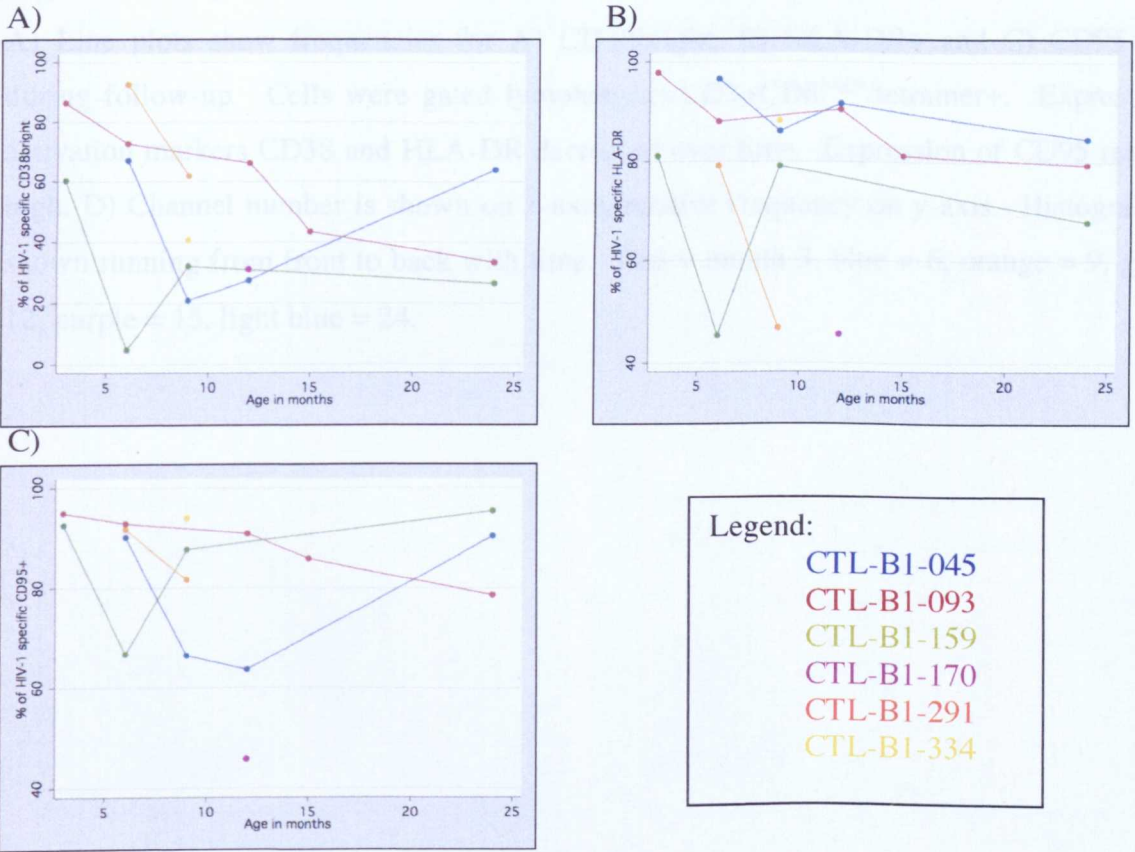


Figure 6.6. Expression of activation markers on HIV-1 specific CD8 T cells.

A) Line plots show frequencies for A) CD38^{bright}, B) HLA-DR⁺ and C) CD95⁺ cells during follow-up. Cells were gated lymphocytes/CD3+CD8^{bright}/tetramer⁺. Expression of activation markers CD38 and HLA-DR decreased over time. Expression of CD95 remained high. D) Channel number is shown on x-axis, relative frequency on y-axis. Histograms are shown running from front to back with time. Red = month 3, blue = 6, orange = 9, green = 12, purple = 15, light blue = 24.

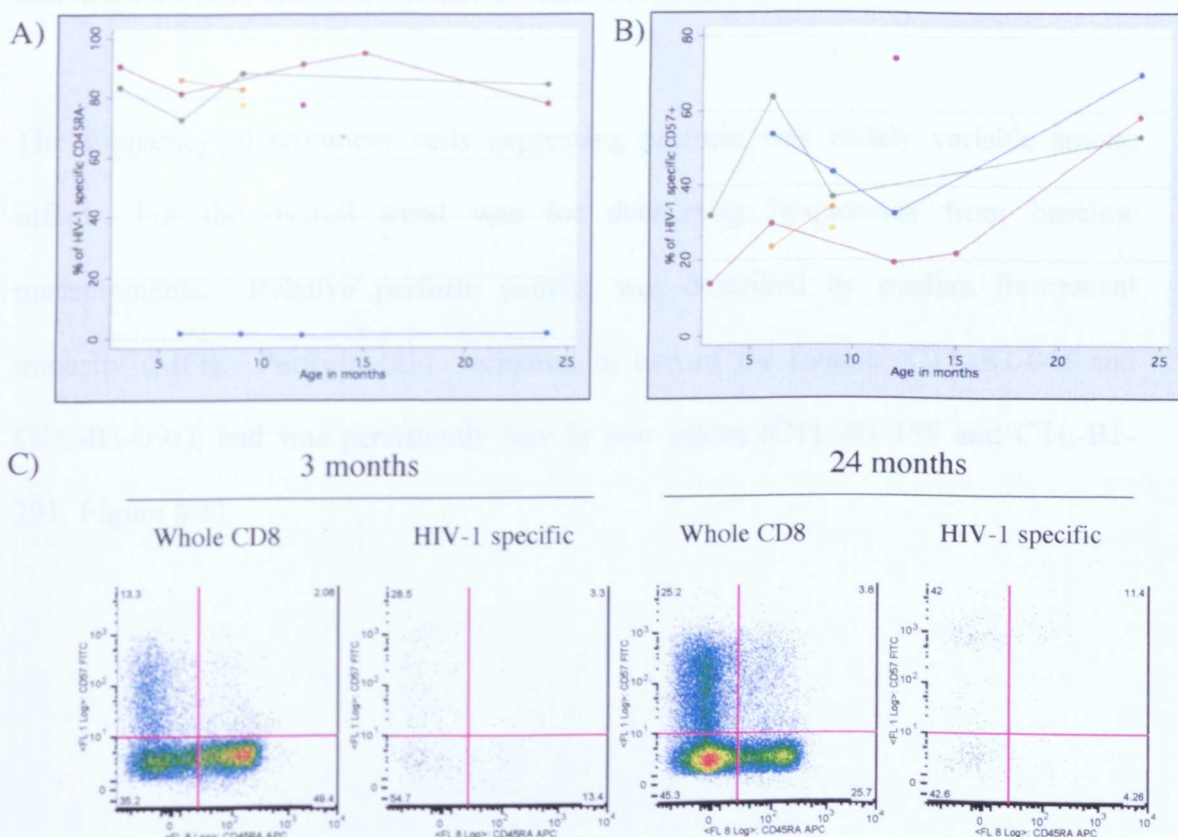


Figure 6.7. CD45RA+ and CD57+ cells in HIV-1 specific CD8 T cells.

A&B) Line plots show frequencies for CD45RA- and CD57+ cells during follow-up. Cells were gated lymphocytes/CD3+CD8^{bright}/tetramer+. Individual infants are indicated by colour. (See legend in Figure 6 for colour key.) In most infants, the majority of HIV-1 specific cells were CD45RA- throughout follow-up, except for CTL-B1-045 (blue line). CD57+ expression increased over time. C) Representative staining from infant CTL-B1-159 is shown at 3 and 24 months of age. Cells were gated lymphocytes/CD3+CD8^{bright} or lymphocytes/CD3+CD8^{bright}/tetramer+. CD57+ cells were primarily CD45RA- but a subset were also CD45RA+ in both the whole CD8 and HIV-1 specific subsets.

frequencies increased in the HIV-1 specific cells, indicating an accumulation of non-replicating antigen-specific cells. CD57+ cells accumulated within CD45RA positive and negative cells in both the tetramer+ and whole CD8 subset.

The frequency of tetramer+ cells expressing perforin was widely variable among infants, but the overall trend was for decreasing frequencies from baseline measurements. Relative perforin content was described by median fluorescent intensity (MFI). Perforin MFI decreased in two of the infants (CTL-B1-045 and CTL-B1-093), and was persistently low in two others (CTL-B1-159 and CTL-B1-291, Figure 6-8).

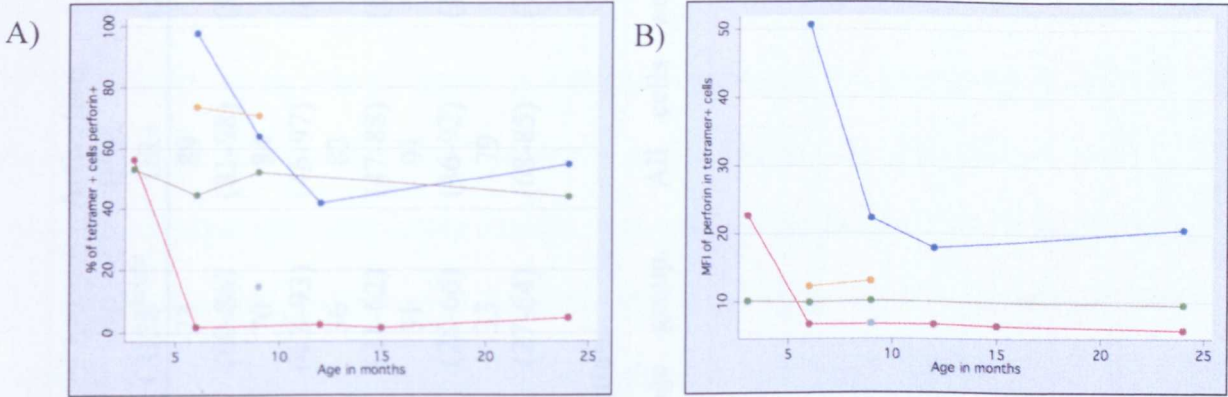


Figure 6.8. Perforin content is low in HIV-1 specific CD8 T cells.

Lines show A) frequencies of perforin+ cells as a % of tetramer+ and B) perforin staining MFI of tetramer+ cells. Individual infants are indicated by colour, see Figure 6 for key.

Age (N)	^a Perforin	Co-stimulation			Memory & Senescence		CD38 ^{bright}	Activation	
		CD27+ CD28+	CD27+ CD28-	CD27-CD28-	CD45RA+	CD57+		HLA- DR+	CD95+
3 (2)	55 (53-56)	29 (26-31)	65 (56-74)	4.9 (0-9.7)	13 (9.5-17)	23 (14-32)	73 (60-86)	89 (81-98)	94 (93-95)
6 (4)	59 (1.8-98)	28 (9.5-35)	66 (53-91)	0 (0-23)	23 (14-98)	42 (23-64)	70 (4.8-93)	84 (46-97)	91 (67-93)
9 (4)	58 (15-71)	17 (6.7-22)	75 (43-83)	3.3 (2.2-48)	20 (12-98)	36 (28-44)	36 (21-62)	83 (47-88)	85 (67-94)
12 (3)	42 (2.8-79)	16 (13-28)	67 (60-84)	9.5 (0.33-19)	22 (8.8-99)	32 (19-74)	31 (28-66)	91 (46-92)	64 (46-91)
24 (3)	44 (4.8-55)	30 (25-30)	68 (66-69)	4.2 (1.3-5.6)	22 (16-98)	59 (53-70)	33 (27-64)	79 (68-85)	90 (79-95)

Table 5. Summary of HIV-1 specific CD8 T cell phenotypes during primary perinatal HIV-1 infection.

Medians (range) are shown for each age. N = number of infants measured in age group. All cells were gated lymphocytes/CD3+CD8^{bright}/tetramer+. ^aPerforin staining was not performed on CTL-B1-170.

Discussion

HIV-1 follows an aggressive course in infants characterised by persistently high levels of viraemia, rapid CD4 decline and a high risk of death. In adults, the reduction of HIV-1 viral load and subsequent control during clinical latency are controlled by CTL (481) (261) (421). HIV-1 specific CD8 responses are detected less frequently in infants and reduced effector function is proposed to explain their poor control of viral load. The generation of fully functional CD8 T cells in combination with CD4 T cell help has been strongly associated with long term survival in HIV-1 infected adults and children. A subset of children with strong IFN- γ ELISpot responses were examined with tetramer staining to determine if T cell frequency and phenotype were comparable to what has been reported in adults undergoing acute HIV-1 infection.

High frequencies of tetramer-stained cells were detected in infants with strong IFN- γ ELISpot responses. The frequency of HIV-1 specific CD8 T cells identified by tetramer staining is in the range of other reports in infants and young children (79, 231, 357, 488) and is the range observed in adults during acute and post-acute infection (22, 402, 412). The decrease observed over time cannot be attributed to clearance of viraemia as all of the infants maintained very high viral loads ~1 million copies/ml. Conversely, the decrease in tetramer-stained cells may be due to increased apoptosis following persistent viral stimulation. Virus has not yet been sequenced from the children, so it is not possible to comment on CTL escape as factor in declining frequencies of tetramer-stained cells.

Overall, there was good concordance between the presence of a positive ELISpot and a population of tetramer-stained cells. Previous studies have demonstrated that not all tetramer+ cells have the ability to produce IFN- γ upon re-stimulation (79, 489). The observation that ELISpot responses in the CTL cohort became stronger with time (333), and tetramer+ cells decreased suggests that a greater proportion of HIV-1 specific T cells are able to make IFN- γ with age, even if their overall numbers are reduced. Unfortunately, limited specimen availability did not enable the simultaneous evaluation of tetramer staining and intracellular cytokine staining for IFN- γ . Tetramer and ELISpot performed here are not directly comparable for a number of reasons. First, ELISpots were performed on freshly isolated PBMC and reflect frequencies of cells expressed as a proportion of PBMC. Ratios of CD4, CD8 and NK cells in blood change rapidly during early life (130), so the proportional numbers of CD8 input into ELISpot assays may change. Secondly, tetramer staining was performed on cryopreserved cells gated to eliminate dead cell debris and NK cells. Thus the parent population from which the tetramer+ cells derive is thus different from that in the ELISpot. Finally, epitopes selected for tetramer evaluation were chosen based on strong IFN- γ responses, so by default high frequency responses are selected. However some insights can be gained from group comparisons of the methods. High levels of persistent viraemia did not result in the maintenance of high frequencies of HIV-1 specific CD8. The high activation status of these cells and expression of CD95 suggests they were likely to be highly vulnerable to apoptosis, and this probably accounts for the decrease in frequency observed with age.

Persistently high HIV-1 viral loads were observed in these children, despite relatively strong IFN- γ responses and high frequencies of tetramer cells. Studies in adults have shown high

frequencies of tetramer+ cells during acute infection (481) (583) followed by a negative correlation between tetramer+ cells and HIV-1 RNA viral load (402). The quality of the T cell response in terms of mature effector function (defined by cytokine production and cytotoxicity) (45) (291) (79) (514) and sustained CD4 help (90, 450) is more closely linked with control of disease and survival than the frequency of HIV-1 specific cells alone.

The phenotype of tetramer+ cells changed over time in the infants. As demonstrated in Chapters 4 and 5, CD38 is constitutively expressed at higher levels in infants, and declines with age. At the first point of detection, cells were CD27+CD28-, highly activated (CD38^{bright}HLA-DR+CD95+), CD45RA- and contained high levels of perforin. In subsequent months, CD27+CD28-, CD45RA-, and CD95+ cells continued to predominate the HIV-1 specific population. Expression of activation markers declined, though they continued to be maintained on the majority of cells during the period of observation. Cells became increasingly CD57+ and low in perforin, suggesting a transition to a senescent phenotype but not necessarily loss of cytotoxicity or IFN- γ (68, 313). These changes are very similar to what has been observed during acute infection of adults (26, 412) ⁴⁶. The infants in the study were only followed for 24 months, and the persistently high viral loads (~1 million copies/ml) maintained during that time make comparisons with chronically infected adults difficult to interpret. Adults and older children with chronic HIV-1 infection have tetramer positive cells exhibiting a more “resting” (lower CD38, HLA-DR, CD95+, perforin^{dim}) phenotype and are similarly CD27+CD28- and senescent (28, 90, 412). However, the downward trend in activation markers and perforin, and increase in CD57+ observed in the current cohort suggests a similar move toward more resting phenotype similar to those detected in adult and older children. From the limited number of markers examined here, it is

not possible to conclude that infant HIV-1 specific CD8 T cells develop a co-stimulatory, activated, or senescent phenotype during acute infection that is different from adults. The current study suggests that IFN- γ production in the cells could be impaired in very young infants, as these responses improved after 1 month of age.

Summary

In summary, the current study of infants during acute and early HIV-1 infection show a similar frequency and pattern of phenotypic changes that are similar to that observed in adults undergoing acute and early HIV-1 infection. T cell phenotype as ascertained by cell surface markers does not explain the failure of infant CTL to control HIV-1 replication.

Chapter 7 New methods

Section 1: Synthesis of the novel HLA-A*2902 tetramer

&

**Section 2: Optimisation and validations for multicolour flow
cytometry**

Section 1. Synthesis of the novel HLA-A*2902 tetramer

Introduction

Tetramer technology was developed in the 1990s as a method to study antigen-specific CD8 T cells directly *ex vivo* (22). Before the advent of tetramer technology, limiting dilution analysis (LDA) was the gold standard used to quantify antigen-specific CD8 cells. In LDA cells are stimulated with antigen, serially diluted, expanded in culture for two weeks and then assessed for lytic activity with a chromium release assay. Precursor cell frequency is then estimated by extrapolation from the lytic assay and dilution. This method underestimated CTL frequencies because it required cells to survive and repeatedly divide in culture for an extended period of time, and assumed that all the dividing cells maintained cytolytic function. Fresh lytic assays performed on cells from patients with HIV-1 infection suggested that antigen specific CD8 frequencies were much higher than estimates from LDA (84, 211). This suggested that only a subset of the antigen-specific CD8 survived the *in vivo* expansion. Similarly, functional assays measuring cytokine release *ex vivo* from patients recovered from influenza virus suggested very high effector memory cell frequencies (305). Direct comparison of LDA, tetramer and intracellular cytokine staining confirmed that the expansion of antigen-specific CD8 during acute viral infection and memory responses were higher than revealed by LDA (385).

Tetramer technology works by exploiting the high specificity of the TCR for its MHC-peptide partner. Tetramers contain four class I heavy chain HLA monomers with recombinant biotinylation tails, each refolded with a viral peptide of 9-11 amino

acids long. The addition of streptavidin or extravidin, both of which have 4 biotin binding sites, allows for the joining of the monomers into “tetramers”. *In vivo*, HLA molecules expressed on the cell membrane have a very fast dissociation rate from TCRs, but “tetramerisation” increases the binding affinity of class I HLA for its TCR ligand, stabilising the interaction. The use of extravidin/streptavidin bound dyes (such as PE or APC) allow tetramers to be identified by fluorescence. In addition to the quantification of epitope-specific cells, multicolour flow cytometry further expands the applications of tetramer staining to enable descriptions of cell surface phenotype, function, and TCR expression (29, 403).

Class I tetramers have revolutionised the study of host immune responses, allowing the direct visualisation of T cells targeting single epitopes. Despite their utility, tetramer technology has limitations. Because the construction of tetramers requires *a priori* knowledge of HLA type, epitope sequences, and immunogenicity, their application is restricted to the study of pre-identified HLA types and epitopes. For example, a large number of HIV-1 epitopes have been described, but most of these are derived from subtype B responses and are recognised by a limited set of HLA alleles common in Caucasian cohorts. By comparison, African class I HLA alleles and immunodominance patterns against HIV-1 subtypes A and D are less well defined. Construction of tetramers is expensive and laborious, and the end product is not always stable in storage. Consequently, although many HIV-1 epitopes have been defined, a limited number of tetramers are currently available for the study of HIV-1-specific immune responses.

HLA types were determined for all infants and mothers in the CTL cohort, and subjects were screened with ELISpot assays using individual epitopes matched for their class I HLA types. HLA-A*2902 was common in the cohort, 57 of the 451 HLA-typed children carried at least one A*2902 allele (12.6%), and the gp120-FNCGGEFFY epitope (582) elicited strong IFN- γ responses in 5 of the 8 HIV+ A*2902 children who were screened with ELISpot assays. This epitope has been reported to elicit strong CTL responses in some HIV-positive donors, but variants are common and changes to the glycine at position 4 have been shown to greatly decrease CTL recognition (582). The possession of HLA-A*2902 has also been associated with increased risk of vertical HIV-1 acquisition (164) and rapid disease progression (236). This section describes the synthesis of a novel HLA-A*2902 tetramer for the study of FNCGGEFFY specific CD8 responses in the CTL cohort.

Methods and results

*Generation of the recombinant HLA-A*2902 heavy chain clone*

RNA was extracted from a B cell line from a Kenyan patient with the HLA-A*2902 allele using the nucleospin kit (Clontech Laboratories Inc., Mountain View California, USA). cDNA was reverse transcribed using AMV reverse transcriptase (Invitrogen Corp., Carlsbad, California, USA). Primers were a gift from Dr. Tao Dong. The beginning of the first class I HLA exon is G-C rich and forms loops that can inhibit translation. The sense primer was designed to prevent secondary structure formation and improve translation. A restriction enzyme sequence for *NcoI* was added to the 5' start of the 5' sense primer (5'-ATATATCCATGGGGCTCCCACTCCATGAGGTATTTAC-3') and a *BamHI*

sequence was added to the 3' end of the antisense primer (5'-ATATATGGATCCCCCATCTCAGGGTGAGGGGCTTGGGCAG-5') to enable cloning. *Pfu* DNA polymerase (Stratagene, LaJolla California) was used to maximise PCR fidelity. The patient's two HLA alleles A*0201 and A*2901 are very similar in sequence at the 5' sense primer site. In order to ensure preferential amplification of the A*2902 allele, the sense primer was designed such that the 3' terminus of the primer was specific for A*2902 and not A*0201, and a high stringency PCR program was used to amplify the gene:

96°C-60s

(96°C-25s, 70°C-45s, 72°C-120s) x 5 cycles

(96°C-25s, 65°C-50s, 72°C-120s) x 21 cycles

(96°C-25s, 55°C-60s, 72°C-120s) x 4 cycles

72°C-120s

The PCR product was excised from a 1.5% agarose gel and purified using the Qiagen Gel Purification Kit (Qiagen Ltd., Crawley, UK). Approximately 250 ng of purified DNA was cut with 2 units of *Bam*HI and *Nco*I in a double digest (Fermentas International Inc., Burlington, Canada) with *Bam*HI buffer and supplemented with BSA) for 4 hours at 37°C. The buffer was exchanged by passing the reaction mixture through a Qiagen Gel Purification kit column and resuspending in water.

A pET23 expression vector was used for the cloning. A previously constructed vector containing a different HLA heavy chain (HLA-A*2401) was used for the cloning because this construct contains the same restriction enzyme recognition sequences and a biotinylation sequence (GGT GGT GGT CTG AAC GAT ATT TTT) that enables construction of the tetramer. The heavy chain insert was removed using

*Bam*HI and *Nco*I and purified using the Qiagen Gel Purification kit as described above.

The ligation of heavy chain HLA-A*2902 DNA and 50 ng of the open pET23 vector was performed with 1:3 and 1:1.5 vector to insert molar ratios using quick T4 DNA ligase (New England Biolabs, Inc.). A negative control (vector with no insert) was included to check the frequency of spontaneous re-ligation. Ligation products were directly transformed into TOP10 competent cells (Invitrogen Corp.) and grown overnight on LB agar plates with 50 µg/ml ampicillin. Ten colonies were selected from the 1:1 plate and grown in 5 ml of LB supplemented with 50 µg/ml ampicillin overnight. Plasmid DNA was purified from the cultures using the Qiagen Miniprep Kit. Approximately 1 µg of DNA was cut using *Bam*HI and *Nco*I as previously described, and run on an agarose gel to confirm the presence of intact vector and insert in all 5 clones (Figure 7.1).

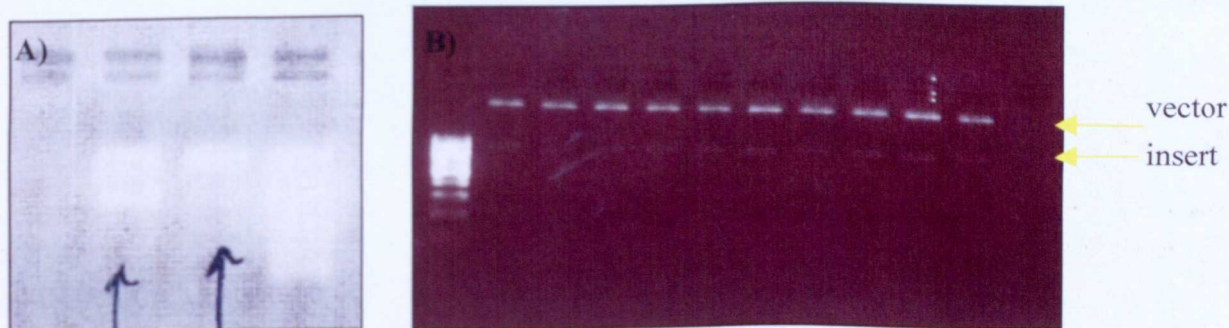


Figure 7.1. Generation of recombinant HLA-A*2902 heavy chain.

A) PCR amplification of cDNA. First two lanes = complete PCR product loaded on gel for extraction. B) Restriction of pET 23-HLA-A*2902 ligation reaction with *Bam*HI and *Nco*I to verify vector/insert sizes.

The clones were sequenced to confirm HLA identity and to ensure the biotinylation signal sequence was intact. DNA sequencing reactions were performed in the MRC DNA sequencing facility with primers recognising the vector (sense primer T7 and antisense rT7). The cloned sequences were confirmed as HLA-A*290201 by running a BLAST search against the EMBL-EBI IMGT/HLA Database (<http://www.ebi.ac.uk/imgt/hla/>, Figure 7.2).

```

>IMGTHLA:HLA00086 A*290201
Length = 1095

Plus Strand HSPs:
Score = 3964 (600.8 bits), Expect = 5.6e-176, F = 5.6e-176
Identities = 796/800 (99%), Positives = 796/800 (99%), Strand = Plus / Plus

Query:   27 ACATCCGTGTCCCGGCCCGGCCGGGGAGCCCCGCTTCATCGCCGTGGGCTACGTGGAC 86
      |||
Sbjct:  100 ACATCCGTGTCCCGGCCCGGCCGGGGAGCCCCGCTTCATCGCCGTGGGCTACGTGGAC 159

Query:   87 GACACCGCAGTTTCGTGCGGTTTGACAGCGACGCCCGGAGCCAGAGGATGGAGCCGCGGGCA 146
      |||
Sbjct:  160 GACACCGCAGTTTCGTGCGGTTTGACAGCGACGCCCGGAGCCAGAGGATGGAGCCGCGGGCA 219

Query:  147 CCGTGGATAGAGCAGGAGGGGCCGGAGTATTGGGACCTGCAGACACGGAATGTGAAGGCC 206
      |||
Sbjct:  220 CCGTGGATAGAGCAGGAGGGGCCGGAGTATTGGGACCTGCAGACACGGAATGTGAAGGCC 279

Query:  207 CAGTCACAGACTGACCGAGCGAACCTGGGGACCTGCGCGGCTACTACAACCAGAGCGAG 266
      |||
Sbjct:  280 CAGTCACAGACTGACCGAGCGAACCTGGGGACCTGCGCGGCTACTACAACCAGAGCGAG 339

Query:  267 GCCGGTTCTCACACCATCCAGATGATGTATGGCTGCGACGTGGGGTCGGACGGGCGCTTC 326
      |||
Sbjct:  340 GCCGGTTCTCACACCATCCAGATGATGTATGGCTGCGACGTGGGGTCGGACGGGCGCTTC 399

Query:  327 CTCGGCGGGTACCGCGCAGGACGCCCTACGACGGCAAGGATTACATCGCCTTGAACGAGGAC 386
      |||
Sbjct:  400 CTCGGCGGGTACCGCGCAGGACGCCCTACGACGGCAAGGATTACATCGCCTTGAACGAGGAC 459

Query:  387 CTGCGCTCTTGAGACCGCGCGGACATGCGCGCTCAGATCACCCAGCGCAAGTGGGAGGCG 446
      |||
Sbjct:  460 CTGCGCTCTTGAGACCGCGCGGACATGCGCGCTCAGATCACCCAGCGCAAGTGGGAGGCG 519

Query:  447 GCCCGTGTGGCGGAGCAGTTGAGAGCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGC 506
      |||
Sbjct:  520 GCCCGTGTGGCGGAGCAGTTGAGAGCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGC 579

Query:  507 AGATACTGGAGAACGGGAAGGAGACGCTGCAGCGCACGGACGCCCCCAAGACGCATATG 566
      |||
Sbjct:  580 AGATACTGGAGAACGGGAAGGAGACGCTGCAGCGCACGGACGCCCCCAAGACGCATATG 639

Query:  567 ACTCACCACGCTGTCTCTGACCATGAGGCCACCTGAGGTGCTGGGCGCTGAGCTTCTAC 626
      |||
Sbjct:  640 ACTCACCACGCTGTCTCTGACCATGAGGCCACCTGAGGTGCTGGGCGCTGAGCTTCTAC 699

Query:  627 CCTGCGGAGATCACACTGACCTGGCAGCGGGATGGGGAGGACCAGACCCAGGACACGGAG 686
      |||
Sbjct:  700 CCTGCGGAGATCACACTGACCTGGCAGCGGGATGGGGAGGACCAGACCCAGGACACGGAG 759

Query:  687 CTTGTGGAGACCAGGCTGCGAGGGGATGGAACCTTCCAGAAGTGGGCGTCTGTGGTGGTG 746
      |||
Sbjct:  760 CTTGTGGAGACCAGGCTGCGAGGGGATGGAACCTTCCAGAAGTGGGCGTCTGTGGTGGTG 819

Query:  747 CCTTCTGGACAGGAGCAGAGATACACCTGCCATGTGCAGCATGAGGGTCTGCCCCAGCCA 806
      |||
Sbjct:  820 CCTTCTGGACAGGAGCAGAGATACACCTGCCATGTGCAGCATGAGGGTCTGCCCCAGCCC 879

Query:  807 CTCACCCCTAGATGGGGTCC 826
      |||
Sbjct:  880 CTCACCCCTAGATGGGAGCC 899

```

Figure 7.2. Alignment of clone 3 with HLA-A*290201 sequence reference sequence HLA00086.

The two mismatched bases at the end of the sequence mark the beginning of the *Bam*HI restriction sequence.

Mini-expression to compare protein expression in clones

Three A*2902-PET clones were screened for protein expression. BL21pLysS cells (Invitrogen) were transformed with the A*2902-PET constructs and grown overnight on LB-agar plates supplemented with 50 µg/ml ampicillin. The next day, one colony from each was grown in 2 ml of low salt LB medium (supplemented with 50 µg/ml ampicillin) shaking at 37°C until the OD at 600 nm was 0.3. Protein production was induced by the addition of 0.5 mM IPTG, and cultures were grown for an additional 4 hours. The bacteria were pelleted by centrifugation at 13,000 rpm for 3 minutes and stored at -20°C overnight. The thawed pellets were resuspended in 50 µl of PBS and 25 µl of 3x reducing buffer (50 mM Tris-Cl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heated for 10 minutes at 95°C. The bacterial lysates were run on a 12% SDS-PAGE gel at 30 mA. The gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) for 4 hours and de-stained over night (10% glacial acetic acid, 50% methanol). A*2902 heavy chain protein (46kD) was induced at similar levels in all clones, clone 3 was chosen for large-scale production of A*2902 protein (Figure 7.3).

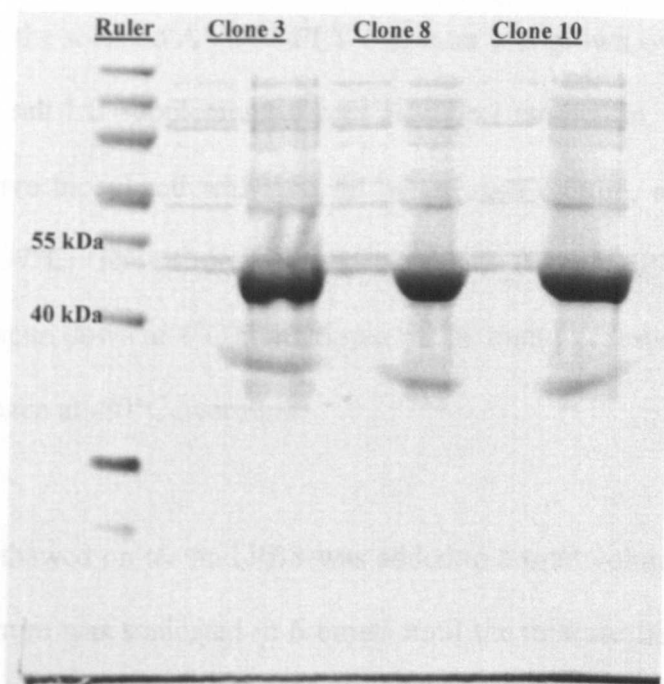


Figure 7.3. SDS-PAGE to compare protein expression of A*2902-PET clones.

Bacterial lysate samples collected before and after induction with IPTG were loaded in the first and second lanes for each clone, respectively.

Protein expression and purification

One colony from the selected A*2902-PET construct was grown overnight, standing, in 1 litre of low salt LB supplemented with 50 µg/ml ampicillin. Six flasks of the same medium were inoculated with 100 ml of the start culture, and grown for 3.5 hours shaking at 37°C. The cultures were induced with 0.5 mM IPTG and grown for another 4 hours, spun down at 4°C at 4000 rpm for 20 minutes, resuspended in 2ml of cold PBS, and frozen at -80°C overnight.

The pellets were thawed on ice and PBS was added to a total volume of 40 ml. The PBS/bacteria mixture was sonicated in 6 bursts until the mixture flowed easily. The lysate was centrifuged for 20 minutes at 15,000 rpm. Remaining bacterial material was removed from the pellet by scraping with a culture loop, and washing with distilled water. The pellet was resuspended in 20 ml of Triton wash (0.5% Triton-X-100, 50 mM Tris pH 8.0, 100 mM NaCl, 0.1% NaN₃, 1 mM EDTA, 1 mM DTT) homogenised, and pelleted by spinning for 15 minutes at 15,000 rpm. The pellet was washed twice more with Triton wash and once with 10 ml resuspension buffer (50 mM Trish pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT). The pellet was then resuspended in 40 ml of urea solution (8M urea, 1M Mes pH 6.5, 0.1 mM EDTA, 0.1 mM DTT), rolled at 4°C for 30 minutes, and centrifuged for 15 minutes at 15,000 rpm to remove precipitate. The Pearce Protein Assay (Bio-Rad Laboratories) was used to estimate the whole protein content of the preparation. Overall, 128 mg of protein was recovered, and stored at 3.2 mg/ml at -80°C.

*Refolding of HLA-A*2902 heavy chain with β_2 microglobulin and gp120 peptide*

β_2 microglobulin ($\beta_2\mu$) protein had been previously prepared using the same method described above. The peptide was synthesised in our laboratory by Dr. Kati DiGleria using F-MOC chemistry. The peptide chosen for refolding is derived from the gp120 region of HIV-1 (HXB2 location: 376-374, sequence: FNCGGEFFY). Refolding buffer (100 mM Tris pH 8.0, 400 mM L arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.1 mM PMSF) was prepared immediately before use and chilled to 4°C. While stirring, approximately 20 mg of peptide (resuspended in PBS at 10 mg/ml) and 10 mg of $\beta_2\mu$ protein were added directly and rapidly to the refolding buffer. Approximately 34 mg of HLA-A*2902 heavy chain protein was added slowly to the mixture, which was left to stir for approximately 24 hours at 4°C. The refolding mixture was spun down at 3000 rpm for 30 minutes at 4°C to remove precipitate. The mixture was concentrated using a stir cell to approximately 50 ml, then to 5 ml using Amicon centrprep centrifugation devices (Millipore Corp., Billerica, Massachusetts, USA). PD-10 desalting columns (Amersham Biosciences, Piscataway, New Jersey, USA) were used to exchange the buffer to (7 ml) 10 mM Tris pH 8.0. The protein was biotinylated at room temperature overnight using a BirA biotinylation kit (Avidity, Denver, Colorado, USA). Leupeptin and pepstatin (1 μ g/ml each) were added to inhibit protease activity.

Purification of protein monomer

The refolded complex was purified using FPLC (Figure 7.4) and eluted into tris-buffered saline (20 mM Tris pH 8.0, 0.6% w/v NaCl). Refolded A29 heavy chain/ β 2 μ /peptide monomer eluted at approximately 150 ml. Free β 2 μ protein eluted at approximately 120 ml and was discarded. Refolded monomer was collected and concentrated to 2 ml. The concentration of the protein was estimated using the Pearce Protein Assay, approximately 4.4 mg total protein was recovered at 2.2 mg/ml. To the final monomer, 1 mM EDTA and 1 μ g/ml each of leupeptin and pepstatin were added. The monomer was stored in 22 μ l aliquots at -80°C.

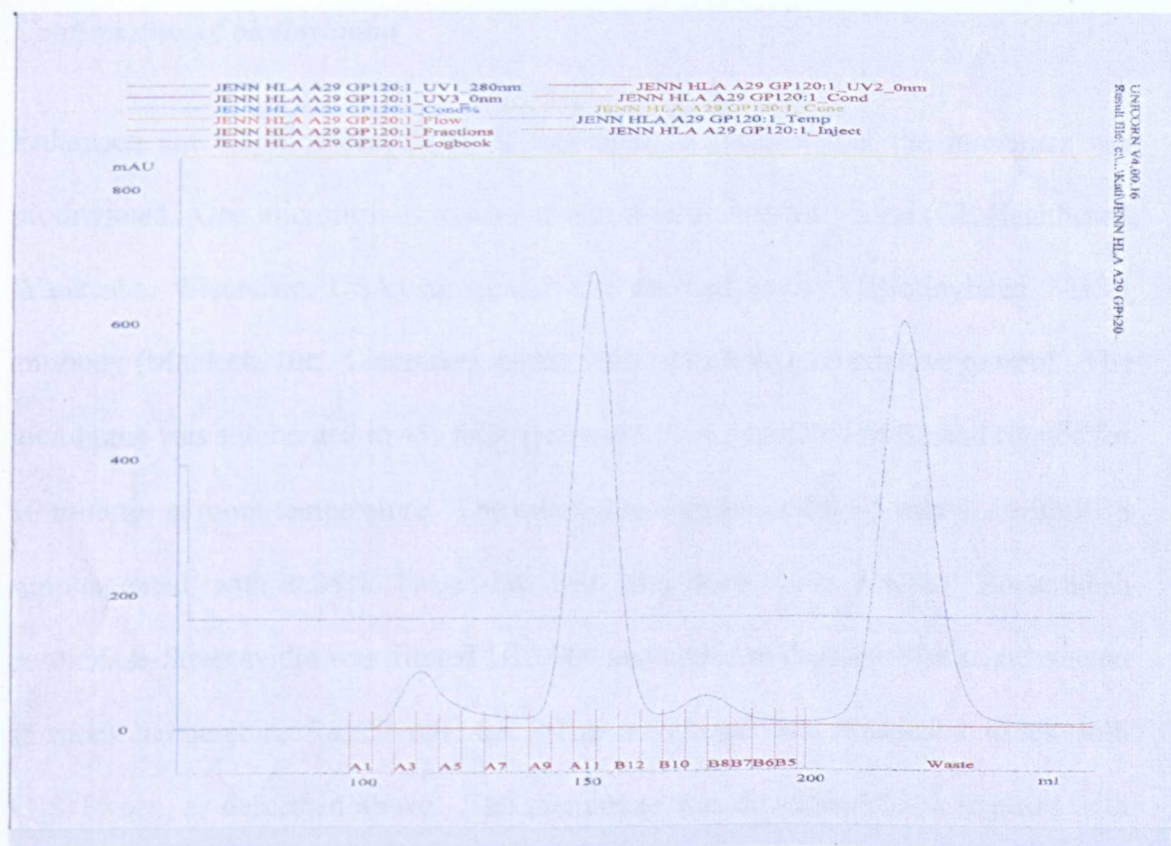


Figure 7.6. FPLC of refolded monomer.

The refolded A29 heavy chain, β 2microglobulin, peptide complex was purified using FPLC (Figure 7.4) and eluted into tris-buffered saline (20 mM Tris pH 8.0, 0.6% w/v NaCl).

Confirmation of biotinylation

Enhanced chemiluminescence (ECL) was used to confirm that the monomer was biotinylated. One microlitre of monomer was blotted onto a Hybond (GE Healthcare, Waukesha, Wisconsin, USA) membrane and allowed to dry. Biotinylated 7-B6-1 antibody (Mabtech, Inc., Cincinnati, Ohio, USA) was used as a positive control. The membrane was submerged in 4% milk (prepared from powdered milk) and rotated for 10 minutes at room temperature. The membrane was washed for 5 minutes with PBS supplemented with 0.05% Tween-20; this was done three times. Horseradish peroxidase-Streptavidin was diluted 1/15,000 and added to the membrane, and rotated at room temperature for 20 minutes. The membrane was washed 3 times with PBS/Tween, as described above. The membrane was developed for 1 minutes with the Western Blot reagents (Amersham) and visualised using Kodak film (Rochester, New York, USA; Figure 7.5).

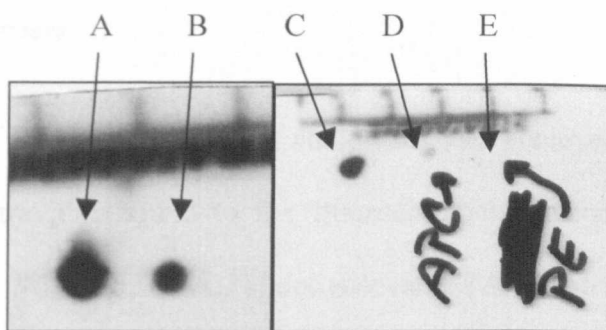


Figure 7.5. ECL to check biotinylation of tetramers.

A&C) Positive control biotinylated antibody 7-B6-1. B) Biotinylated HLA-A*2902 monomer with no extravidin or streptavidin added. HLA-A*2902 tetramer conjugated with D) streptavidin-APC and E) extravidin-PE.

Generation of tetramers

Tetramerisation of refolded monomeric complexes was achieved by the addition of streptavidin or extravidin bound to the fluorochromes phycoerythrin (PE, Sigma Aldrich, St. Louis, Missouri, USA), allophycocyanin (APC, Molecular Probes, Inc., Eugene, Oregon), PE-Cy5 (BD Biosciences Pharmingen, San Diego, California, USA) and Pacific Blue (Molecular Probes). The amount of streptavidin or extravidin stock added was calculated based on the number of moles of monomer and the molecular weight of the streptavidin/extravidin-stock. Streptavidin and extravidin each have 4 biotin binding sites, so a 4:1 molar ratio of streptavidin/extravidin-stock to monomer was added. Stocks were added in small aliquots and ECL was performed at intervals to monitor the amount of unbound biotin remaining. Figure 7.5 shows an example of a tetramer in which the optimal amount of stock has been added (PE labelled); unbound biotin is just barely visible as a faint grey spot. In this figure the APC-biotin sites are not saturated, so the spot is more visible. In most cases, the amount of extravidin/streptavidin needed for biotin saturation was less than the amount calculated based on molarity of the heavy chain. This is probably due to the overestimation of our monomer concentration. A BioRad Pearce assay was used to estimate overall protein concentration, but is likely that small amounts of impurities and misfolded monomer will result in error in concentration measurements. The different labels were tested on different tetramers (previously synthesised in our laboratory) and tested on CTL clones specific for their epitope (CMV-NLV and HIV-B8NEF) to determine which gave the brightest and most specific staining. Extravidin-PE (Sigma) and Streptavidin-APC (Pharmingen) were the only fluorochromes that stained cells brightly and with low background (defined as minimal staining in CD8 negative cells).

Optimisation of A29 tetramer staining

Cryopreserved PBMC isolated from an individual lacking the HLA-A*2902 allele were used as negative controls in the experiments. Control experiments to optimise tetramer staining were performed on cryopreserved PBMC from an HLA-A*2902 infant previously confirmed to have a response to A29 FNC peptide in ELISpot assays. An A29-FNC CTL line was generated from these cells in order to have adequate numbers of cells for control experiments. Cryopreserved cells were thawed in 5ml of FCS and washed twice with RPMI supplemented with 10% human serum (H10) and counted using Trypan Blue to define dead cells. The cells were split and resuspended at a concentration of 0.5 million live cells/ml. One quarter of the cells were stimulated for 1 hour with 10 µg/ml A29 FNC peptide at 37°C. The cells were combined with the unstimulated fraction and suspended in H10 supplemented with 25 ng/ml recombinant IL-7 at a concentration of 2 million cells/ml. Cell lines were cultured at 5% CO₂, 37°C. At 3 days in culture, the media was replaced with H10 supplemented with H10 at 200 U/ml. The cells were checked daily and the media was changed when it became yellow. The cells were split on day 10 when they had become “crowded” in appearance. On day 0 and 14 cells were stained with A29 tetramer.

In all staining experiments, cells were resuspended in approximately 20 µl of R10 media, and tetramer added. The cells were incubated at 5% CO₂, 37°C for 15 minutes. Surface antibodies were added and cells were incubated at room temperature in the dark for 20 minutes. The cells were washed twice and fixed with Cytofix (BD Biosciences Pharmingen). Before acquisition, cells were refrigerated at 4°C at least one hour for the inactivation of HIV-1.

A dilution series was performed to determine the optimal concentration of tetramer to be used in staining. Since cell numbers were limiting even with a CTL line, only two dilutions were performed and HIV-1 negative donor cells were used as a negative control. Previous experience in the laboratory has demonstrated that approximately 0.5 μ g is sufficient to stain as many as 1 million PBMC. Cells were stained with tetramer at 25 μ g/ml, and 50 μ g/ml. There appeared to be no increase in staining with higher concentration of tetramer, so staining with 0.5 μ g of tetramer (25 μ g/ml) was determined to be of sufficient concentration for high levels of staining of CD8 positive cells with low staining of CD8 negative cells. High populations of tetramer-stained cells were found in HIV+ A*2902 subjects, and no stained cells were found in A*2902 negative subjects (Figure 7.6).

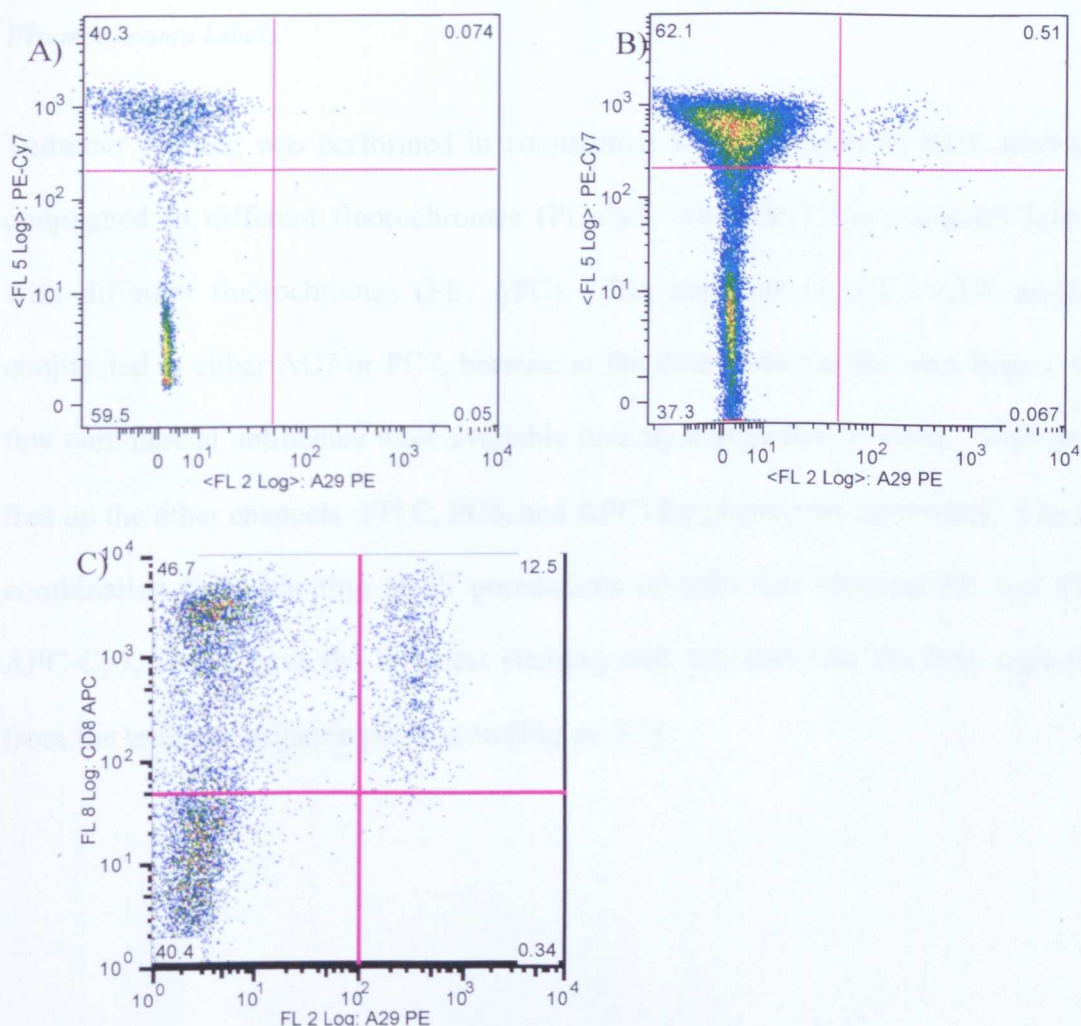


Figure 7.6. HLA-A*2902 tetramer staining.

PBMC from an A) HLA-A*2902 negative patient and an B) HLA-A*2902 positive patient with HIV-1 infection were stained with A29-PE tetramer. C) A CTL line (from the same A*2902 positive patient) shows expansion of tetramer-staining cells at day 14.

Fluorochrome labels

Tetramer staining was performed in conjunction with a number of CD8 antibodies conjugated to different fluorochromes (PE-Cy7, APC-Cy7) and tetramers labelled with different fluorochromes (PE, APC). The aim was to use a CD8 antibody conjugated to either AC7 or PC7, because at the time these studies were begun, very few commercial antibodies were available directly conjugated to these. This would free up the other channels (FITC, PC5, and APC) for phenotypic antibodies. The best combination for separating small populations of cells was tetramer-PE and CD8-APC-Cy7, which gave the brightest staining with tetramer and the best separation from the tetramer-negative population (Figure 7.7).

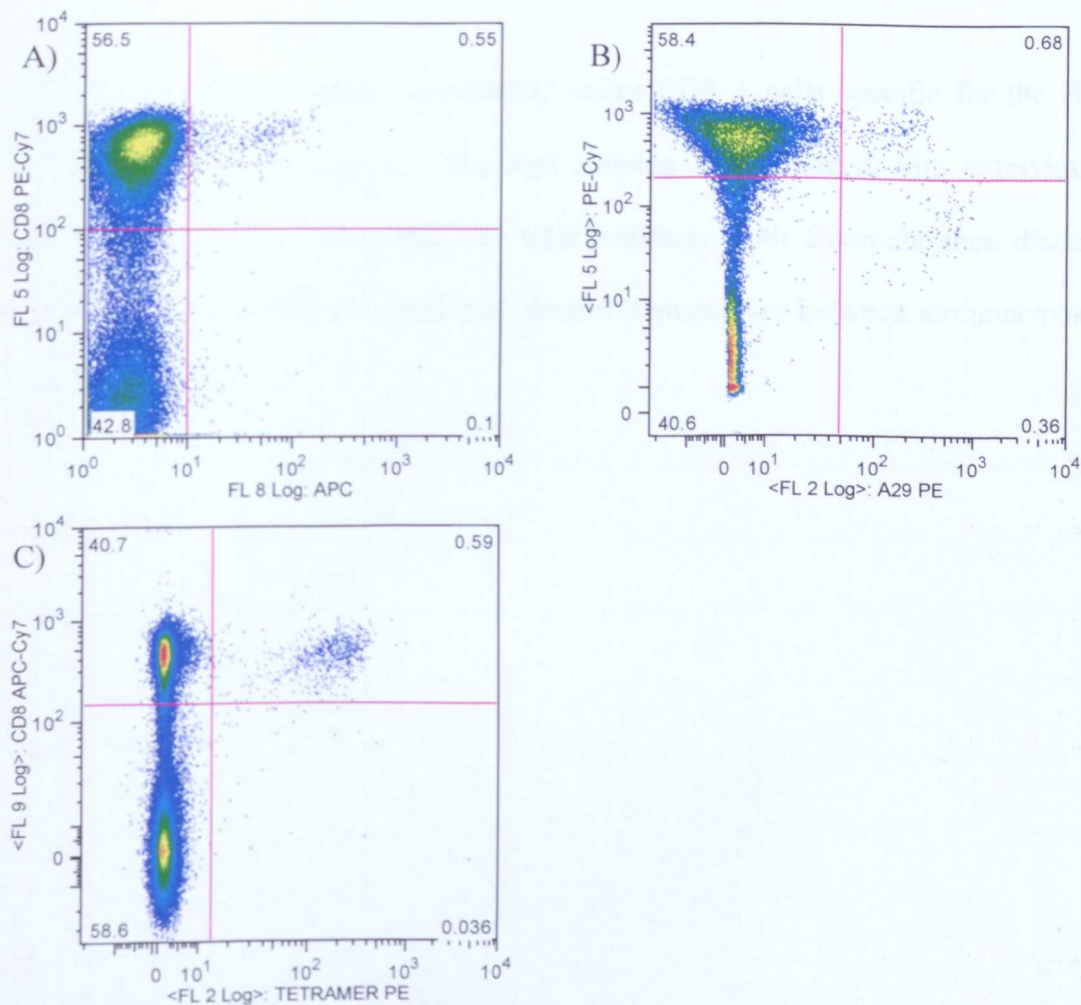


Figure 7.7. Different fluorochromes for tetramer and CD8 staining.

PBMC from an HIV-1 infected patient were stained with HLA-A*2902 tetramer and CD8 conjugated with A) CD8-PC7 and tetramer-APC B) CD8-PC7 and tetramer-PE and C) CD8-AC7 and tetramer-PE.

Summary

The HLA-A*2902 tetramer specifically stains CD8 T cells specific for the HIV-1 HLA-A*2902 FNC epitope. The best staining was obtained with extravidin-PE labelled antibodies. Labelling the CD8 antibody with fluorochromes distant in emission spectra from PE made the clearest demarcation between tetramer positive and negative cells.

Section 2: Optimisation and validations for multicolour flow cytometry

General Introduction

Flow cytometry has been used widely in immunology to describe the phenotype of cells based on their expression of particular antigens. New instruments with the ability to measure more than 4 colours have become common in academic laboratories. Each additional colour utilised increases one's ability to describe lymphocyte populations as more antibodies/stains can be added to an experiment. However, each additional colour also increases the complexity of experimental design and data analysis. Our laboratory had no experience with 9-colour flow cytometry at the inception of this study, so numerous validation experiments were performed prior to the collection of the real study data. This chapter describes the process of validation and optimisation of the flow cytometry experiments.

The main aims of these control experiments were:

- Optimisation of experimental protocols
- Reduction and monitoring of experimental error
- Precision, consistency and objectivity in gating of lymphocyte populations
- Development of management and analysis systems for large volumes of flow cytometry data

Are data collected on two different instruments comparable?

All experiments described in this thesis were performed on Dako Cytomation CyAn instruments (Dako Cytomation, Angel Drove, UK). Two CyAn models were used in the study, the ADP and the LX. Both models have the same configuration base configuration and optics, the LX has an improved red filter and lower red laser output. The CyAn is fitted with 3 lasers: 405 nm Solid-State Violet Diode, 488 OPSL Sapphire, and 635 nm Red Diode. This configuration enables an 11-parameter system of detection: FSC, SSC, and detectors FL1 through FL9.

At the beginning of this study, the WIMM was in possession of a Dako Cytomation CyAn ADP. Approximately a year into the study this instrument was replaced with Dako's CyAn LX, and it was necessary to perform the remaining experiments with the updated model. In order to use data from experiments acquired on the CyAn ADP and LX, it was necessary to determine whether there were differences in the measurements produced by the two instruments. Experiments were run concurrently on both instruments to estimate variation between the measurements.

Methods

Cells from a healthy laboratory donor (Healthy Donor = HD) were stained with antibody panels A-F, and simultaneously acquired on both machines. Both instruments were independently configured to optimal photomultiplier tube (PMT) voltage settings and compensation values. Following acquisition, FCS files were transferred to a single analysis computer, and analysed independently in separate FlowJo workspace groups. FCS files were auto-compensated with FlowJo's software

using single stain controls. For each panel, cells were gated for lymphocytes on SSC/FCS. CD3⁺ cells were gated against SSC, then CD4⁺ and CD8^{hi} single positive cells were selected as sub-gates of CD3⁺. A separate gating path was made for CD3⁻CD56⁺ cells on the SSC/FSC lymphocyte gate. These gates applied to each panel of antibodies so the base subsets (CD3, CD4, CD8, NK) would be the same; permeabilised cells were gated as their own group separately because their autofluorescence changes after permeabilisation. CD4⁺ and CD8^{hi} cells were sub-gated for further analysis with guidance from isotype controls. Within the subset gates, cells in each panel were gated for PE⁺ APC⁺ and FITC⁺ staining. Panel G was excluded from this part of the analysis because it is largely redundant to the other panels.

Results

Comparison of LX and ADP frequency estimates

Cells stained with panels A-F were gated: Lymphocyte/CD3⁺/CD4⁺ and Lymphocyte/CD3⁺/CD8^{hi} and analysed for FITC, PE, and APC positive staining. Data collected from the ADP and LX instruments fit a near perfect linear model with a slope of 1 (Figure 7.8, slope = 1.00, R square = 0.96, $p < 0.0001$).

FITC, PE, and APC measurements were also considered as separate groups using linear regression. These models also showed very good linear correlation: FITC model: R square = 0.97, slope = 0.90, $p < 0.0001$ PE: R square = 0.95, slope = 1.1 $p < 0.0001$ APC: R square = 0.99, slope = 0.99, $p < 0.0001$.

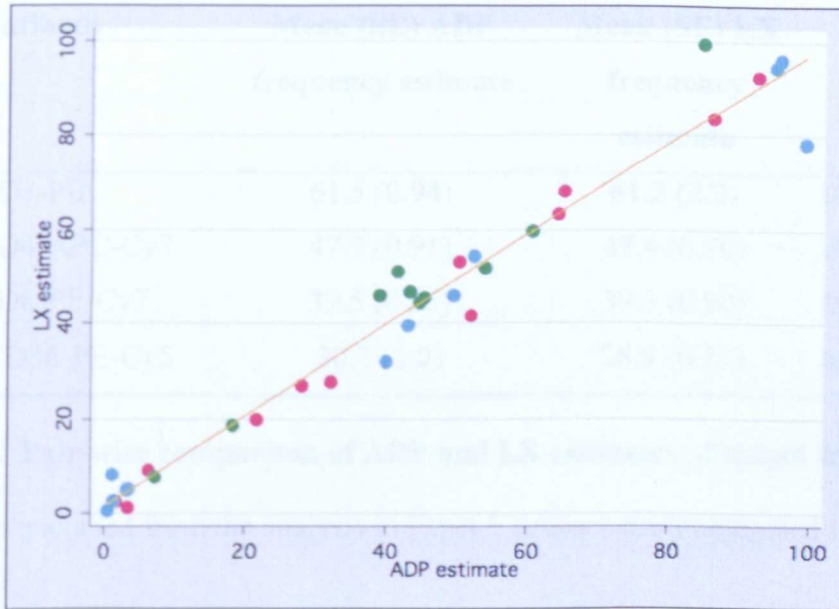


Figure 7.8. Comparison of cell frequencies obtained from ADP and LX flow cytometers.

All observations are shown as a scatter plot, with linear regression line overlaid for the entire set of observations (slope = 1.00, $p < 0.0001$). Blue = FITC, green = PE, red = APC.

CD3-Pacific Blue (PB), CD56-PE-Cy5, CD4-APC-Cy7 and CD8-PE-Cy7, were included in multiple panels. Pair-wise T tests were used to compare group means estimated by the different instruments. No significant differences were found between frequency estimates for PB, PE-Cy5, PE-Cy7 and APC-Cy7 on the different machines (all $p > 0.05$, Table 7.1).

Antibody	Mean (SE) ADP frequency estimate	Mean (SE) LX frequency estimate	P
CD3-PB	61.5 (0.94)	61.2 (2.2)	0.83
CD4-APC-Cy7	47.7 (0.91)	47.4 (0.70)	0.82
CD8-PE-Cy7	39.5 (0.85)	39.3 (0.90)	0.91
^a CD56-PE-Cy5	30.2 (1.0)	28.9 (0.73)	0.49

Table 7.1. Pair-wise comparison of ADP and LX estimates of subset frequencies.

^aPE-Cy5 is excluded from the analysis in Panel F because it is conjugated to CD152.

Comparison between LX and ADP estimates of fluorescence intensity

Channel statistics (mean, median, standard deviation) are often used in flow cytometry to evaluate relative density of the target antigen's expression on a cell. Mean fluorescence intensity (MFI) of a fluorochrome is the most commonly used statistic, it refers to the mean channel number of signals received on the 1024 channels of a flow cytometer. However MFI must be used with caution because it is extremely vulnerable to experimental error; undersaturation of epitopes, excess antibody, adjustments in PMT voltage between experiments, and autofluorescence can modify MFI. By comparison, frequency estimates are more robust. Moving from the ADP to the LX instrument was a potential source of experimental error for the current study. MFIs were examined to determine whether this measurement error was consistent and could be used to adjust for differences between machines.

Although the LX and ADP flow cytometers returned similar frequency estimates, the MFIs were different. An example of cells acquired on the LX and ADP generated

FCS files is shown in Figure 7.9. These dot plots show cells stained with Panel D, CD27-APC is shown along the y-axis, CD4-APC-Cy7 is shown along the x-axis. Though the frequency of CD27+ cells gated was similar, the MFI of APC was significantly higher when measured on the ADP compared to the LX. This difference may be due to the improved red-filters of the LX. Photomultiplier tubes (PMTs) logarithmically amplify incoming signals so they can be visualised by the user as dot plots. PMT voltages are adjusted by the user such that stained cells can be resolved into positive and negative populations. A higher PMT voltage was applied to resolve APC-stained cells acquired on the ADP instrument.

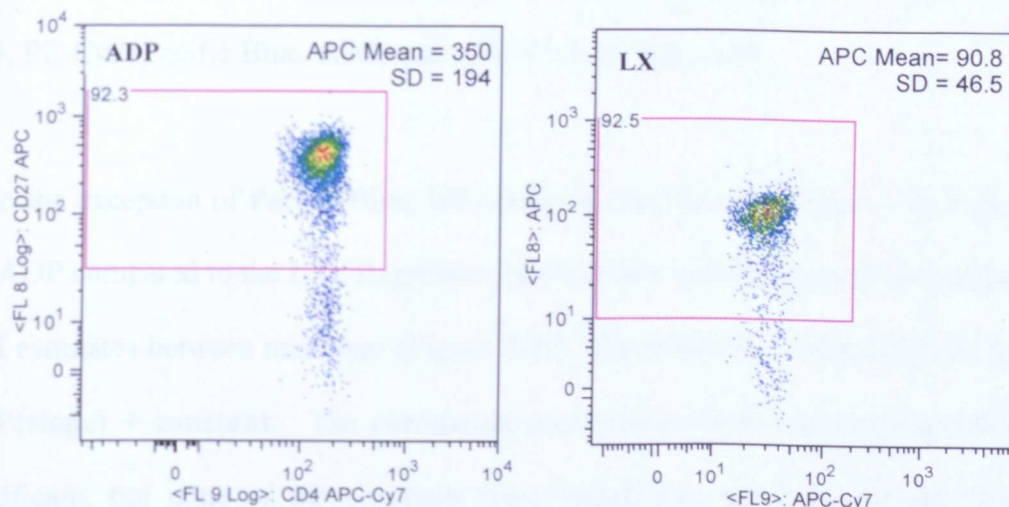


Figure 7.9. Cells stained with the same antibodies have different MFI measurements on the ADP and LX flow cytometers.

Lymphocytes were gated on CD3+/CD4+ cells and CD27 gating was determined with the aid of isotype controls. Though the estimated frequency of the CD27+ cells is similar between the instruments, the channel statistics of APC are different.

MFIs collected from HD cells on the LX and ADP were compared using linear regression. Lymphocytes were gated, and MFIs were estimated for FITC, PE, PE-Cy5, PE-Cy7, Pacific Blue, APC, and APC-Cy7 in Panels A-F.

With the exception of Pacific Blue, MFI estimates for fluorochromes were higher on the ADP compared to the LX. Regression showed near perfect linear relationships for MFI estimates between machines (Figure 7.10). The model fit to the data was: **LX = ADP(slope) + constant**. The correlation coefficients (slope) in each model were significant, but none of the constants were significant; the equation can thus be simplified to **LX = ADP(slope)**. The correction for FITC was 0.47, PE 0.59, APC 0.27^a.

^a Ultimately, I decided not to use MFI in comparisons between infants because it is so vulnerable to experimental error. I used MFI to show differences between different samples run on the same day (eg, B1-00x specimens from birth, month 1, etc.). However, even these should be interpreted with caution as differences in cell numbers of different specimens may mean different antibody saturation between staining wells.

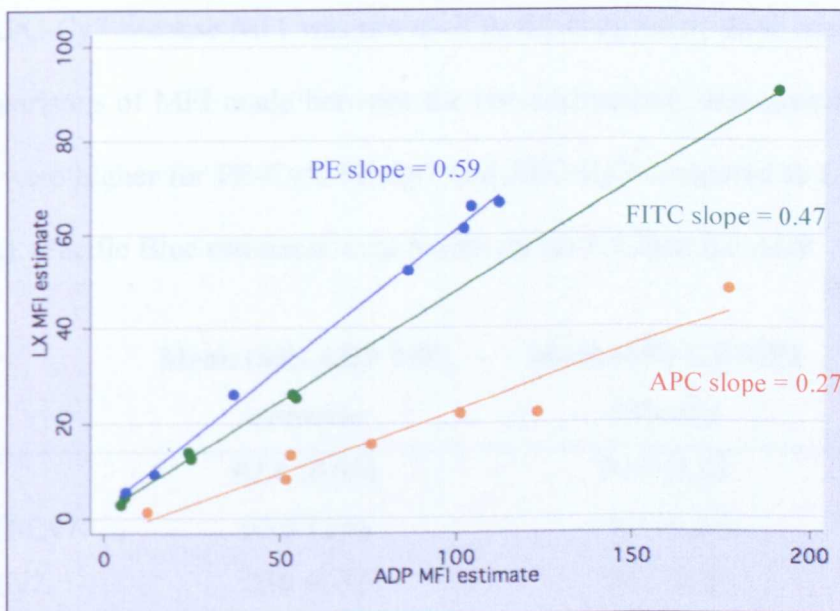


Figure 7.10. Linear models for MFIs estimated on the LX and ADP instruments.

There was a near-perfect linear correlation between MFI estimates from the ADP and LX instruments. For all fluorochromes, the slopes in these models were significant ($p < 0.0001$) but the constants were not ($p > 0.05$) and were thus excluded in the model. The transformation $[\text{ADP} \times \text{slope}]$ can be used to transform ADP estimates before comparison with LX data. Linear fits: FITC: R square = 0.99, $p < 0.0001$, PE: R square = 0.99, $p < 0.0001$, APC R square = 0.92, $p = 0.0004$.

Adjustments were not necessary for CD3-Pacific Blue, CD56-PE-Cy5, CD8-PE-Cy7 or CD4-APC-Cy7 because MFI was not used in the analysis of these markers. Pair-wise comparisons of MFI made between the two instruments demonstrate that ADP estimates were higher for PE-Cy5, PE-Cy7 and APC-Cy7 compared to LX estimates (Table 7.2). Pacific Blue estimates were higher on the LX than the ADP.

Antibody	Mean (SE) ADP MFI	Mean (SE) LX MFI	P
	estimate	estimate	
CD3-PB	62.0 (0.95)	90.9 (2.0)	< 0.0001
^a CD4-APC-Cy7	63.9 (2.6)	11.6 (0.36)	< 0.0001
CD8-PE-Cy7	250 (4.5)	89.1 (2.3)	< 0.0001
^b CD56-PE-Cy5	47.5 (3.1)	20.8 (1.2)	0.0001

Table 7.2. Pair-wise comparison of ADP and LX estimates of MFIs.

Cells were gated on lymphocytes before generating channel statistics.

^aAPC-Cy7 is excluded in this analysis from Panel G because its conjugated to CD16.

^bPE-Cy5 analysis is excluded form Panel F because it is conjugated to CD152.

Discussion

Direct comparison of data collected from two different Cyan models revealed differences between the two instruments. Frequency estimates of cell populations were similar between the two instruments for all of the fluorochromes tested, but MFI estimates were not. This is an important observation, particularly for long-running studies that may utilise multiple instruments, or upgrade to a newer model, as was the case with the current study.

Simple modelling with linear regression revealed that the equation $LX = ADP \text{ (slope)}$ can be used to transform data for comparison between the two instruments used in this study. A simple linear equation may not be appropriate in all cases, and

comparisons should be made on the basis of individual instruments to determine the appropriate model. Alternately, single stain controls or fluorescently labelled beads can also be used to monitor differences between instruments or changes due to upgrades or maintenance work. This monitoring should be considered prospectively before beginning a study.

Titration of antibodies

Antibodies were titrated in order to optimise resolution of discrete cell populations. The goals of titration were to achieve antibody saturation of antigen and to maximise signal-to-noise ratio (SNR). Cryopreserved cells from HD were thawed and stained with single antibodies. Approximately 300K cells were used per stain. Single antibody was added to cells at varying volumes: 1, 3, and 5 μ l. If saturation was not reached by 5 μ l, the experiment was repeated with 1, 3, 5, 7, 9 and 10 μ l.

Each antibody used in the study was titrated using single stains of PBMC. Neat volumes of antibody added were varied, rather than concentration, because in the laboratory's experience the best staining is achieved with the most concentrated antibody/cell mixtures. Figure 7.11 shows an example of the titration process. Small lymphocytes were selected according to FSC/SSC. MFI was plotted for positive and negative populations. The goal was to achieve saturation of positive staining cells, but to avoid adding excess antibody, which may result in non-specific adherence to negative cells. Graphically, over-saturation can be visualised when the cells gated positive begin to decrease and the cells gated negative begin to increase in MFI (Figure 7.11C); cells from the negative gate begin to move into the lower range of the positive gate, causing the overall MFI of the positive gate to decrease. Optimal antibody volume was identified as the volume *before* the MFI of negative populations began to increase significantly and positive MFI levelled off or began to decrease.

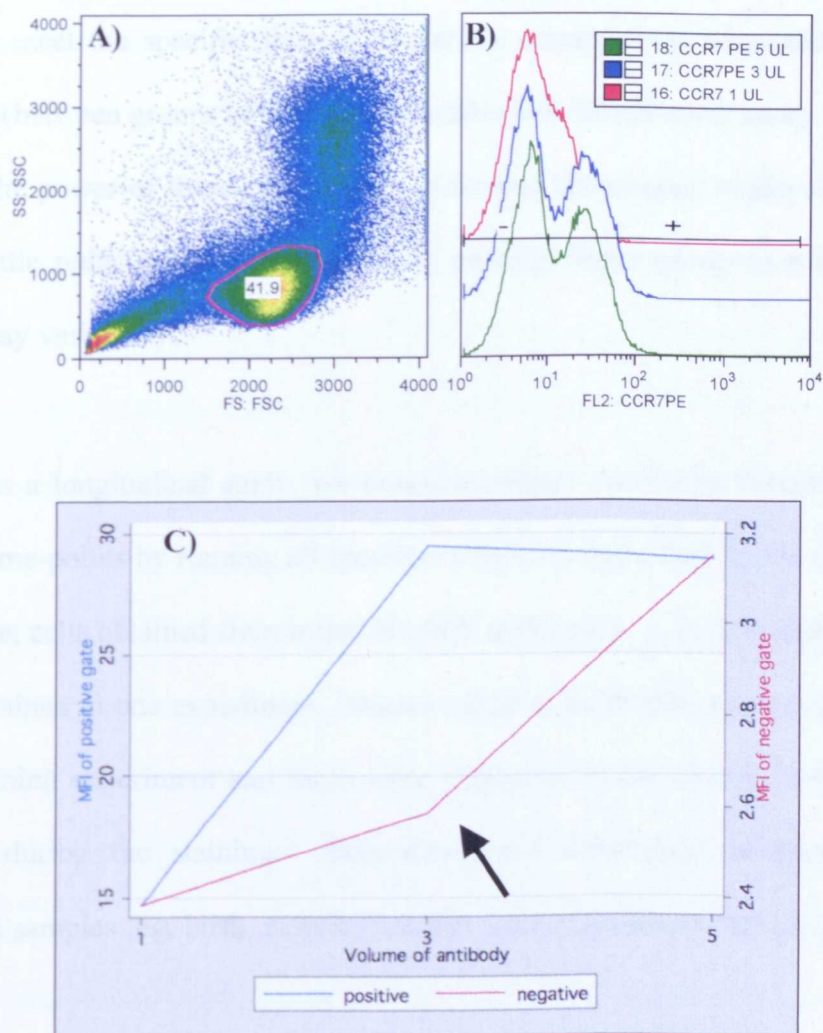


Figure 7.11. Titration for CCR7-PE antibody.

Approximately 300K cells were stained with 1, 3, or 5 μ l of CCR7-PE antibodies. A) Lymphocytes were gated based on size and granularity. B) Lymphocytes gated for positive (+) or negative (-) staining with CCR7-PE antibody. C) MFI of positive (blue) and negative (red) gates plotted against volume. Arrow indicates the optimal volume chosen for future experiments.

Controlling and monitoring of experimental error

In order to meet the specific aims of this study, comparisons were made *between* individuals (between groups of infants) and *within* individuals (over time). In order to maximise the power of these experiments to resolve differences within and between subjects while reducing experimental error, controls were set up to minimise and monitor assay variability.

Since this is a longitudinal study, we aimed to reduce variability between different specimen time-points by running all specimens from an individual in one experiment. For example, cells obtained from infant B1-00X at delivery, 1, 3, 6, 9, and 12 months would be stained in one experiment. Master mixes of antibodies were prepared prior to each staining experiment and these were aliquotted in the appropriate amount to each well during the staining. This minimised intra-assay variation between longitudinal samples (eg, birth, month 1, month 3 etc) specimens stained on the same day.

Reduction and monitoring of inter-experiment and inter-patient variability

This study is examining a large number of specimens, so it was impossible to run all of the specimens in the same experiment. In order to minimize inter-experiment variation, PMT voltage settings were not altered between experiments (which would affect MFI). Compensation was checked and modified for each experiment; adjustments were rarely needed. FlowJo software was used to re-compensate after each acquisition; FlowJo uses a fixed compensation algorithm so this was chosen as the most consistent and unbiased method available.

Controls were put in place so that time-dependent changes in instruments (Eg., temperature of optics, laser wandering, instrument maintenance) could be monitored. At a single time point, 100 million cells were isolated from a healthy lab donor (HD), and split into many separate vials and stored in liquid nitrogen. These cells were used as controls in all the phenotyping experiments. For each experiment, HD were stained with single antibodies labelled with FITC, PE, PE-Cy5, PE-Cy7, PB, APC and APC-Cy7. HD cells were also used for unstained and isotype negative controls. Since the same source sample was used in each experiment, it was possible to monitor variation in antibodies and the instrument over time.

Quality control

The tandem conjugate antibodies were unstable and degraded over a period of weeks when frequently used. Aliquotting of these into separate dark vials at the time of receipt somewhat extended the lifespan of these antibodies, presumably by reducing the amount of light exposure (which causes fluorochrome degradation) and evaporation (which causes changes in pH). PE-Cy5 antibodies were comparatively the most stable (> 6 months). APC-Cy7 and PE-Cy7 were much less stable, with a lifespan of approximately 3 months once opened. FITC/PE and APC antibodies were very stable by comparison and showed no evidence of declining quality within 6 months to a year of opening.

Tandem conjugates were tested in single stains on a weekly basis before staining CTL study samples. Full antibody panels were tested on HD cells approximately every 6 months. This process allowed antibody degradation to be detected in time to prevent inclusion in an experiment.

An example of antibody degradation is shown in Figure 7.12. As the bond is broken between the PE and Cy7 molecules, one is left with what is essentially a PE-antibody, and doubly positive (PE+ and PE-Cy7+) cells appear in dot plots. The antibody shown represents an unusual case as the PE-Cy7 degraded very quickly (over days, rather than months, and was quickly and apologetically replaced by the vendor) but it is an excellent example for this discussion. At time day 0, when the antibody was first opened, it had been aliquotted into separate dark vials and stored at 4 °C. On day 2 and 8, the cells were stained again with the antibody (both from the same aliquot). On day 8, cells were additionally stained with antibody from an unopened aliquot

from day 0. By day 2, a separate population of PE-stained cells becomes visible. By day 8, the antibody has degraded to the point where the majority of stained cells are doubly positive for PE and PE-Cy7. The unopened aliquot from day 0 also showed some degradation.

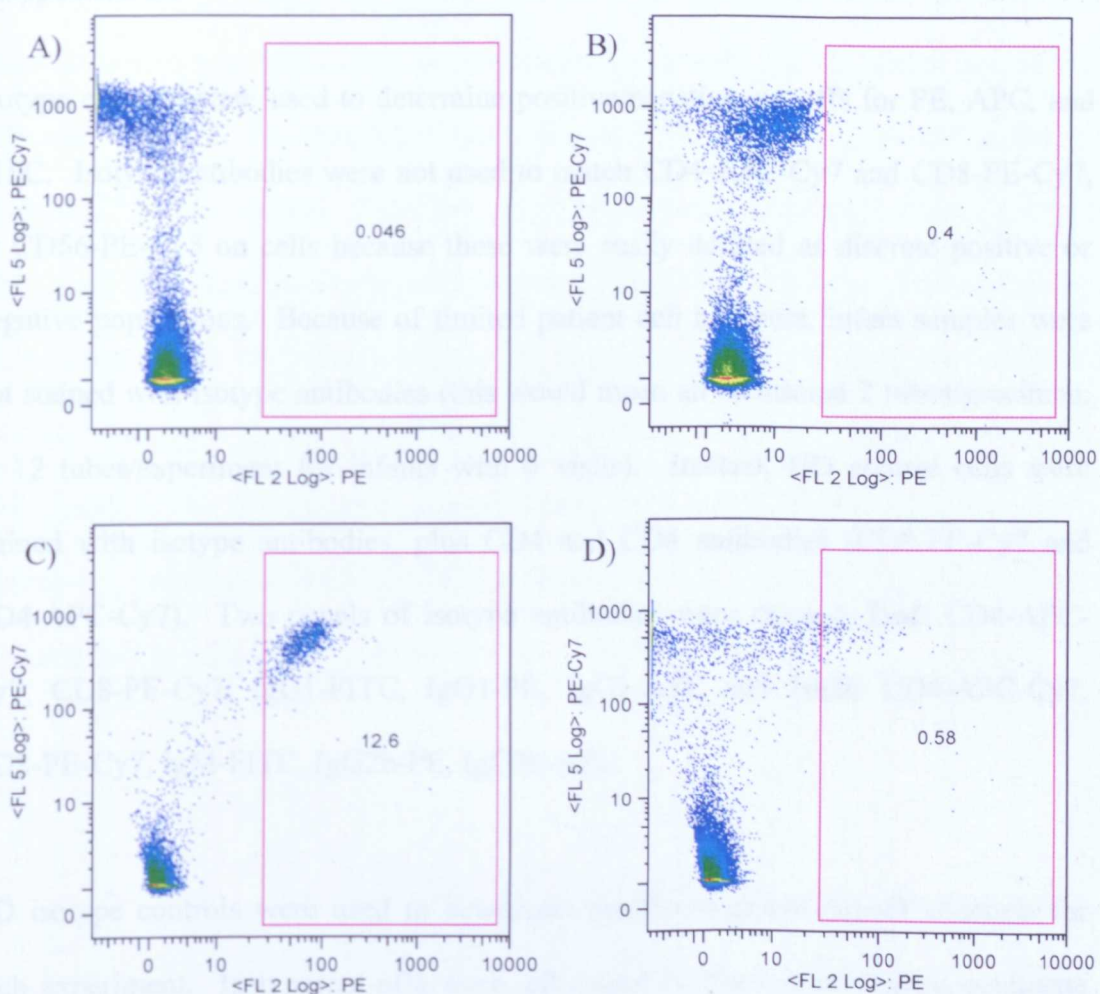


Figure 7.12. Illustration of PE-Cy7 degradation.

Cells were stained only with CD8-PE-Cy7, gate frequencies show percentage of cells positive for PE staining. Figure A shows “normal” staining pattern with a low percentage of cells staining for PE and most PE-Cy7 positive cells aligned with the negative population or close to the y-axis. Two days later B) the percentage of cells staining with PE is higher and the entire PE-Cy7+ population has shifted to a higher MFI. C) Eight days after the initial stain the PE staining has become so high that it cannot be compensated. D) Shows staining on day 8 from an aliquot preserved and unopened from day 0, which shows, less, but still visible degradation.

Isotype controls

Isotype controls were used to determine positive/negative cut-offs for PE, APC, and FITC. Isotype antibodies were not used to match CD4-APC-Cy7 and CD8-PE-Cy7, or CD56-PE-Cy5 on cells because these were easily defined as discrete positive or negative populations. Because of limited patient cell numbers, infant samples were not stained with isotype antibodies (this would mean an additional 2 tubes/specimen, or 12 tubes/experiment for infants with 6 visits). Instead, HD control cells were stained with isotype antibodies, plus CD4 and CD8 antibodies (CD8-PE-Cy7 and CD4-APC-Cy7). Two panels of isotype antibodies were created, **IsoI**: CD4-APC-Cy7, CD8-PE-Cy7, IgG1-FITC, IgG1-PE, IgG1-APC and **IsoII**: CD4-APC-Cy7, CD8-PE-Cy7, IgM-FITC, IgG2b-PE, IgG2b-APC.

HD isotype controls were used to determine positive/negative cut-off channels for each experiment. Isotype cut-offs were influenced by the use of tandem conjugate antibodies. Tandem conjugate antibodies such as PE-Cy7 have two emission spectra: one for the primary conjugate (PE) and one for the tandem (Cy7, which is amplified by energy from the excited PE-molecule). This is illustrated in the histogram in Figure 7.13, which compares detection in FL2 (PE) of cells stained with nothing, PE-Cy5, PE-Cy7 or PE. This “extra peak” must be considered in setting cut-offs to distinguish positive and negative cell populations. Setting the cut-off correctly becomes especially important when measuring molecules such as CD69 or CD71, which are expressed at low density on the cell surface. Mixing the isotype antibodies with the CD4/CD8 tandem conjugates allows one to set a threshold that takes into account the PE and APC emission within PE-Cy7 and APC-Cy7. The PE peak is larger for PE-Cy7 than PE-Cy5, and so the PE cut-off channel was determined by the

PE peak of PE-Cy7 rather than that of PE-Cy5. Practically, this meant that different channels sometimes marked the positive/negative cut-offs for the same isotype on CD4 and CD8 cells. An example of this is shown in Figure 7.13. Cells were gated by CD4⁺ or CD8^{hi} expression. The CD4 and CD8^{hi} subsets were then examined for PE, APC, and FITC staining. The positive/negative cut-off was set as the channel number where stained cells constituted <1% of the gated population. Cells gated on CD4-APC-Cy7 require a higher cut-off channel to distinguish positive/negative stains for APC (channel 24.4) compared to cells gated on CD8^{hi}-PE-Cy7 (channel 10.1). Alternately, cells gated on CD8^{hi}-PE-Cy7 (channel 20.3) require a higher cut-off for PE compared to cells gated on CD4-APC-Cy7 (channel 3.9).

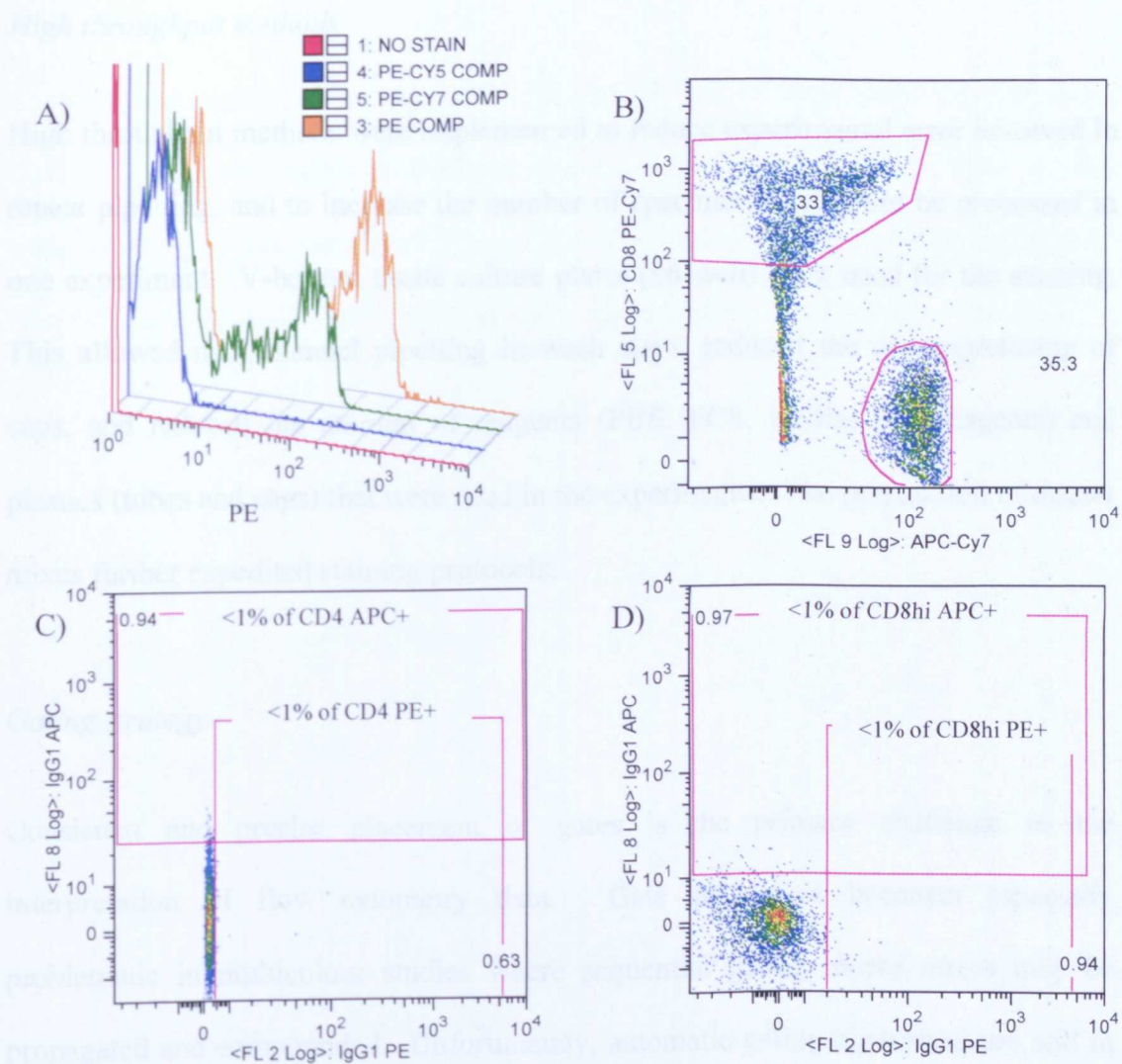


Figure 7.13. Example of isotype controls to aid in the setting of positive/negative cut-offs.

A) Histogram showing PE fluorescence in cells stained *singly* with nothing (red), PE-Cy5 (blue), PE-Cy7 (green) or PE. Weak PE emission is observed in tandem conjugates due to fluorescence of the PE molecule. B) The first step in setting positive/negative cut-offs is to select single positive populations for CD4 (APC-Cy7) and CD8 (PE-Cy7 bright). The second step is to set gates such that less than 1% of cells stains positively for isotype controls within C) CD4 and D) CD8 subsets. Because of the PE and APC peaks from tandem conjugates, different PE and APC cut-offs are sometimes set for PE-Cy7 and APC-Cy7 gated cells.

High throughput methods

High throughput methods were implemented to reduce experimental error involved in repeat pipetting, and to increase the number of specimens that could be processed in one experiment. V-bottom tissue culture plates (96-well) were used for the staining. This allowed multichannel pipetting in wash steps, reduced the opening/closing of caps, and reduced the amount of reagents (PBS, FCS, media, ICS reagents) and plastics (tubes and caps) that were used in the experiments. The preparation of master mixes further expedited staining protocols.

Gating strategy

Consistent and precise placement of gates is the primary challenge in the interpretation of flow cytometry data. Gate placement becomes especially problematic in multicolour studies where sequential gating means errors may be propagated and compounded. Unfortunately, automatic gating algorithms are still in their infancy – they require a dense number of cells to be well separated from other populations, and are unable to resolve negative, low frequency or diffuse cell populations.

The aim of the current study was to make comparisons between individuals and within individuals over time. The following algorithm was used in gating of the data:

- 1) Set baseline of isotype control (set negative cut-off of <1% positive staining with isotype) as in Figure 7.13. Do this separately for CD4 and CD8.
- 2) Apply gates to stained cells, copy to all visits so they are the same
- 3) Check gates, shift to higher channels if gates bisect relevant populations.

Figure 7.14 illustrates an example of the gating process. For the majority of samples, isotype gates appropriately defined populations without further modification. In this example, isotype samples are used to set cut-offs for positive and negative staining of CD38-PE and HLA-DR-APC, and these are applied to all of the patient's samples.

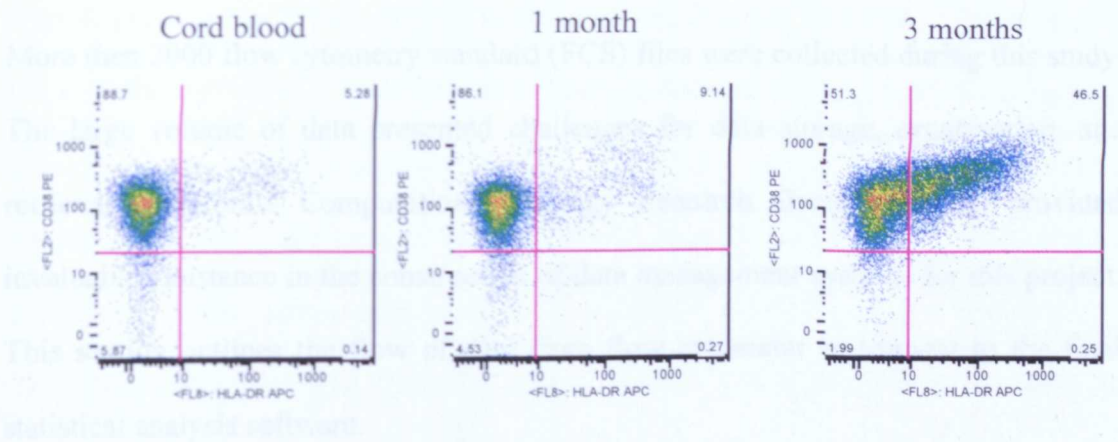


Figure 7.14. Placement of gates on 2-dimensional dot plots.

Cells were gated lymphocytes/CD3+/CD8hi. Isotypes were used to set positive/negative cut-offs for PE and FITC, the gates were then applied to all of the infant's specimens.

Data management system

More than 2000 flow cytometry standard (FCS) files were collected during this study. The large volume of data presented challenges for data storage, organisation, and retrieval. Oxford's Computational Biology Research Group (CBRG) provided invaluable assistance in the construction of data management systems for this project. This section outlines the flow of data from flow cytometer instrument to the final statistical analysis software.

Processing of FCS files

FCS files were collected on the CyAn flow cytometers. The size of FCS files depends on the number of events acquired (cells counted), the number of lasers used, and the number of parameters collected. For the multicolour experiments, a minimum of 50,000 cells and a maximum of 1 million cells were acquired in each file, file sizes ranged from about 1-20 MB. The total memory volume taken up by the FCS files was 20.5 GB.

The large file sizes collected in this study restricted options for analysis software. Summit (Dako) software was used for data acquisition on the flow cytometers, but was not suitable for the analysis of large or multiple files. FlowJo (Tree Star, Inc. Olten, Switzerland) was selected for analysis because it was the only software available at the time that allowed easy batch analysis of large numbers of samples of large file size. Following the software developer's recommendations, FlowJo v.8.5.3 software was run on a MacPro dual-core processor with 3 GB of RAM.

FCS files were compensated with FlowJo, organised into groups based on antibody panels, gated, compiled as FlowJo tables, and exported as text files containing cell frequencies and MFIs. These text files were re-formatted using a PERL (Practical Extraction and Report Language) script. The PERL script automates important functions that allow the data to be more easily accessible to the user, for example, complex FCS file names such as CTL-B1-001-CORD-PANEL-A are broken down into separate variables (*STUDY* (CTL or MIP), *CTLCODE* (mother or baby), *IDNUM*, *VISIT*, *PANEL*) based on the “-” delimiter. Data is also transposed by the script such that 100+ columns of data are reduced to 15 columns of data (by creating grouping variables), which greatly facilitates downstream movement and analysis of data. The reformatted files are then uploaded into a laboratory information management system (LIMS) database for further management (Figure 7.15).

All_Specs_Panels_errorsfix_jot-E														
Accession Subject Value Type Foot	SDATE	STOT	Lymphocytes Freq. of Parent	Lymphocytes CD3+ Freq. of Parent	Lymphocytes/CD3+ CD4 Freq. of Parent	Lymphocytes/CD3+ CD4 SD <FL1> CD69 FITC	Lymphocytes/CD3+ CD4 Median <FL1> CD69 FITC	Lymphocytes/CD3+ CD4 Mean <FL1> CD69 FITC	Lymphocytes/CD3+ CD4 Geom. Mean <FL1> CD69 FITC	Lymphocytes/CD3+ CD4 Mean <FL2> CD38 PE	Lymphocytes/CD3+ CD4 Median <FL2> CD38 PE	Lymphocytes/CD3+ CD4 Mean <FL2> CD38 PE	Lymphocytes/CD3+ CD4 Median <FL2> CD38 PE	Lymphocytes/CD3+ CD4 Mean <FL2> CD38 PE
1-26: PANEL E MIP B-110 CORO	07-Apr-05	561827	27.1	71.1	45.5	3.81	3.88	4.96	3.89	187	177	187	177	187
2-29: PANEL E MIP B-122 CORO	07-Apr-05	7745	40	57.2	66.3	0.9	1.57	1.75	1.6	159	176	159	176	159
3-30: PANEL E MIP B-122 M6	07-Apr-05	146	56.7	72.4	64.4	0.87	1.64	1.81	1.66	147	129	147	129	147
4-31: PANEL E MIP H-110 M6	07-Apr-05	146	21.6	64.2	61.6	1.04	1.92	2.1	1.91	17	34.6	17	34.6	16.9
5-32: PANEL E MIP H-122 M6	07-Apr-05	685165	12.9	65.7	55.8	1.05	1.93	2.1	1.92	13.7	10.6	13.7	10.6	13.7
6-25: PANEL E CTL B1-JHO CORO	09-Apr-05	96357	42.9	18.9	64.5	1.65	2.18	2.46	2.15	139	157	139	157	139
7-26: PANEL E CTL B1-JHO M1	09-Apr-05	128523	50.6	56.1	66	1.73	1.85	2.02	1.83	85.1	88.1	85.1	88.1	85.1
8-27: PANEL E CTL B1-JHO M3	09-Apr-05	644115	39.1	46.5	45.1	1	1.82	1.97	1.8	49.6	51.9	49.6	51.9	49.6
9-28: PANEL E CTL M-JHO M3	09-Apr-05	146	14.2	64.4	38.9	1.88	1.74	1.97	1.77	21.3	26.2	21.3	26.2	21.3
10-30: PANEL E CTL H-94-081 M6	14-Apr-05	146	23.4	62.3	44.5	1.23	2.19	2.37	2.15	30	27.3	30	27.3	30
11-39: PANEL E CTL B1-081 CORO	14-Apr-05	892525	20.6	39.9	77	1.91	2.75	3.14	2.7	141	160	141	160	141
12-40: PANEL E CTL B1-081 M1	14-Apr-05	251133	45.8	59.2	72.9	1.28	2.29	2.45	2.19	121	147	121	147	121
13-41: PANEL E CTL B1-081 M3	14-Apr-05	226317	47.4	52.8	49.6	1.22	2.29	2.45	2.21	88.4	100	88.4	100	88.4
14-42: PANEL E CTL B1-081 M6	14-Apr-05	257785	92.8	76.5	63.8	1.16	2.25	2.38	2.15	124	144	124	144	124
15-43: PANEL E CTL B1-081 M9	14-Apr-05	209523	68.5	51.4	40.7	1.28	2.33	2.48	2.25	62.5	74.7	62.5	74.7	62.5
16-44: PANEL E CTL B1-081 M12	14-Apr-05	468245	48.1	45.5	41.1	1.34	2.29	2.51	2.25	82	64	82	64	82
17-52: PANEL E CTL B1-135 M1	19-Apr-05	404307	21.7	64.5	53.9	1.52	2.22	2.66	2.26	111	127	111	127	111
18-53: PANEL E CTL B1-135 M3	19-Apr-05	422085	32.5	46.9	29	1.52	2.07	2.35	2.08	76.4	89.6	76.4	89.6	76.4
19-94: PANEL E CTL B1-135 M6	19-Apr-05	444727	39.7	51.6	12.4	1.16	2.06	2.23	2.04	105	105	105	105	105
20-95: PANEL E CTL B1-135 M9	19-Apr-05	342065	54.3	62.4	11.6	1.2	1.92	2.07	1.89	56.4	65	56.4	65	56.4
21-96: PANEL E CTL B1-135 M12	19-Apr-05	215943	41	74.5	7.88	1.47	2.13	2.44	2.16	55.7	61.1	55.7	61.1	55.7
22-37: PANEL E CTL B1-250 M12	28-Apr-05	215479	19.4	67.6	30.8	1.49	2.35	2.79	2.5	64.2	72.5	64.2	72.5	64.2
23-38: PANEL E CTL B1-250 M9	28-Apr-05	305585	64.9	78.7	27.2	1.13	2.22	2.35	2.13	88.7	96.3	88.7	96.3	88.7
24-39: PANEL E CTL B1-250 M6	28-Apr-05	215777	31.1	67.8	35.9	1.35	2.81	2.97	2.7	95.3	114	95.3	114	95.3
25-51: PANEL E CTL B1-250 CORO	28-Apr-05	465203	235	30.7	37	1.89	8.33	5.63	3.2	108	134	108	134	108
26-54: PANEL E MIP B-111 CORO	05-May-05	176131	39.6	48.8	52	1.87	2.05	2.21	2.04	110	144	110	144	110
27-55: PANEL E MIP B-111 M6	05-May-05	295457	14.5	55	58.9	1.12	2.01	2.21	2.06	45.5	57.5	45.5	57.5	45.5
28-56: PANEL E MIP B-119 CORO	05-May-05	596277	13.5	44.3	35.9	1.24	2.08	2.27	2.09	111	149	111	149	111
29-37: PANEL E MIP B-119 M6	05-May-05	146	66	48.7	52.8	0.98	1.73	1.87	1.77	72.8	86.4	72.8	86.4	72.8
30-38: PANEL E MIP H-111 M6	05-May-05	5245	39.1	69.5	39.1	1.05	2.14	2.28	2.15	15.9	35	15.9	35	15.9
31-39: PANEL E MIP H-119 M6	05-May-05	737883	45.7	70.6	64.3	1.02	2.09	2.29	2.12	40.6	12.1	40.6	12.1	40.6
32-47: PANEL E CTL H-102 P32	06-May-05	163413	18.8	81	55.9	1.46	2.03	2.31	2.12	32.7	36.9	32.7	36.9	32.7
33-48: PANEL E CTL B1-105 M1	06-May-05	474147	30.1	65.6	61	2.29	2.63	3.18	2.76	118	150	118	150	118
34-49: PANEL E CTL B1-105 M3	06-May-05	137377	23.7	53.3	33.7	1.86	2.79	3.09	2.8	96.6	110	96.6	110	96.6

Figure 7.15A. Re-formatting of flow cytometry data.

Tables created within FlowJo contain cell frequency and channel statistics. Each cell type evaluated appears as its own column with one row for each file acquired. In this example, Panel E contains 108 columns of data (not all are shown). A PERL script transposes the data such that file names (the left-most column) is broken into variables, and grouping variables are created to transpose the data to 15 columns. The resulting files are imported into the FACS LIMS database to form a new table called **Panel (Figure 7.15B)**.

Figure 7.15B. Re-formatting of flow cytometry data.

FACS LIMS Database

The FACS LIMS database was designed for this study by Stephen Taylor of the CBRG. The database was designed using Microsoft Access (Microsoft Corporation, Seattle, WA). FACS LIMS is a Relational Database Management System (RDMS) containing clinical, laboratory, and research data collected from the cohorts. The tables contained in the database are shown in Figure 7.16. All tables are joined by the variables *STUDY*, *CTLCODE*, *ID*, and *VISIT* which when combined uniquely identify each line of data in a table. This simple relationship allows the user to easily extract and combine data from multiple tables for export as text files.

Name	Description	Modified	Created	Type
Create table in Design view				
Create table by using wizard				
Create table by entering data				
ARV		8/9/2006 2:20:30 PM	8/9/2006 2:13:50 PM	Table
ASSAY_TABLE		7/26/2005 5:11:31 PM	7/26/2005 3:43:17 PM	Table
B1_AGE_HIV		9/18/2006 10:35:0...	8/9/2006 3:35:21 PM	Table
CELLS		8/14/2006 2:30:37 PM	8/14/2006 2:22:35 PM	Table
CELLS Table		8/14/2006 2:11:19 PM	8/14/2006 2:08:58 PM	Table
CTL_MASTER_INVENTORY\$ImportErrors		8/14/2006 1:48:24 PM	8/14/2006 1:48:24 PM	Table
CTL_ASSAY		8/14/2006 2:30:10 PM	8/14/2006 2:23:06 PM	Table
CTL_MASTER_INVENTORY		8/14/2006 1:48:30 PM	8/14/2006 1:48:20 PM	Table
CTL_VIRAL_LOADS		9/13/2006 2:49:39 PM	6/1/2006 3:47:25 PM	Table
DELIVERY		8/9/2006 2:24:12 PM	8/9/2006 2:17:00 PM	Table
ENROLMENT		8/9/2006 2:28:24 PM	8/9/2006 2:26:59 PM	Table
HLA_TYPES		8/14/2006 2:29:12 PM	8/14/2006 2:21:22 PM	Table
INFANT_CD4		9/18/2006 12:04:2...	8/9/2006 2:29:47 PM	Table
INFANT_FOLLOWUP		8/9/2006 2:34:54 PM	8/9/2006 2:34:17 PM	Table
INFANT_IMMUNIZATION		8/9/2006 2:38:17 PM	8/9/2006 2:37:35 PM	Table
INFANT_MORTALITY_CTL		9/13/2006 3:27:13 PM	8/9/2006 2:39:58 PM	Table
LTFU		8/9/2006 2:42:15 PM	8/9/2006 2:41:50 PM	Table
MATERNAL_CD4		9/18/2006 12:04:2...	8/9/2006 2:43:11 PM	Table
MATERNAL_FOLLOWUP		8/9/2006 2:49:24 PM	8/9/2006 2:49:07 PM	Table
MATERNAL_HLA		8/1/2006 10:13:03 ...	6/25/2005 2:15:05 PM	Table
MATERNAL_MORTALITY		8/9/2006 2:50:22 PM	8/9/2006 2:49:56 PM	Table
MAX_CTL_HIV_VL		9/18/2006 10:58:0...	8/9/2006 3:36:58 PM	Table
MAX_CTL_HIV_VL\$ImportErrors		8/9/2006 3:36:58 PM	8/9/2006 3:36:58 PM	Table
MM_BB_HAEMOGLOBIN		8/9/2006 3:06:19 PM	8/9/2006 3:04:57 PM	Table
NAIROBI_INVENTORY		8/1/2006 10:13:30 ...	6/17/2005 4:26:57 PM	Table
NEONATAL_EXAM		8/9/2006 3:07:06 PM	8/9/2006 3:06:50 PM	Table
P32_EXAM		8/9/2006 3:08:03 PM	8/9/2006 3:07:35 PM	Table
PANEL		9/13/2006 2:49:39 PM	6/1/2006 3:58:39 PM	Table
PREGNANCY_MONITORING		8/9/2006 3:08:56 PM	8/9/2006 3:08:41 PM	Table
SET DEFINITIONS		7/26/2005 3:10:33 PM	7/26/2005 3:04:10 PM	Table
tbTest		9/18/2006 9:09:04 ...	9/18/2006 9:09:04 ...	Table
tbTempTest2		9/18/2006 9:14:59 ...	9/18/2006 9:14:59 ...	Table
TREE		6/2/2006 2:23:38 PM	6/1/2006 4:00:02 PM	Table

Figure 7.16. Tables in the FACS LIMS database.

Clinical, laboratory and research data files generated from the cohorts are combined in the FACS LIMS database. Each file appears as a table in the cohort, which is linked to all other tables by the unique identifier combination of variables: *STUDY*, *CTLCODE*, *ID*, and *VISIT*.

The Panel Query (Figure 7.17) forms the centre of the data extraction system for the phenotyping study. This query is organised in a tree-like structure that emulates the gating trees found in FlowJo's workspace files. The Panel Table and its associated query contain all of the frequency, MFI, and other data generated in FlowJo. The query allows the user to extract data from any combination of gates desired and to combine these data with other patient data from the database. The data set generated for analysis can be as large (including all phenotypes) or as small as the user requires. Additionally, the user can easily modify the query's design to add additional data from linked tables (Figure 7.18). The files exported from the database were then analysed using the statistical software STATA (Stata Corp, College Station, TX). STATA analysis programs were written to automate data cleaning, checking, production of tables, and generation of figures. This greatly streamlined the analysis as new samples were tested and added to the existing data set. A summary of the flow of data from the CyAn to the final analysis is illustrated in Figure 7.19.

The screenshot shows a software window titled "frmTREE : Form". On the left, there is a tree view under the heading "LYMPHOCYTES". The tree structure is as follows:

- LYMPHOCYTES
 - CD3+
 - CD4
 - CD8HI
 - CD27+
 - CD28+
 - CD45RA+
 - Q01: CD28 FITC-,CD27 PE+
 - Q02: CD28 FITC+,CD27 PE+
 - Q03: CD28 FITC+,CD27 PE-
 - Q04: CD28 FITC-,CD27 PE-
 - CD45RA-
 - Q01: CD28 FITC-,CD27 PE+
 - Q02: CD28 FITC+,CD27 PE+
 - Q03: CD28 FITC+,CD27 PE-
 - Q04: CD28 FITC-,CD27 PE-
 - Q01: CD28 FITC-,CD27 PE+
CD27- PE
 - Q02: CD28 FITC+,CD27 PE+
 - Q03: CD28 FITC+,CD27 PE-
 - Q04: CD28 FITC-,CD27 PE-
 - CCR7 PE
 - CCR7+
 - CCR7-
 - CCR7HI
 - CD57 FITC
 - CD57+
 - CD57-
 - Q01: CD57 FITC-,CCR7 PE+
 - Q02: CD57 FITC+,CCR7 PE+
 - Q03: CD57 FITC+,CCR7 PE-
 - Q04: CD57 FITC-,CCR7 PE-
 - CD38 PE
 - CD38+
 - CD38-
 - CD69 FITC
 - CD69+
 - HI ΔNR ΔPC

On the right side of the window, there are several buttons:

- Query Viral Load
- Query CD4
- Query HIV AGE PEAK
- Query Infant Mortality
- Query Delivery
- Query All
- Reset Form

At the bottom of the window, there is a status bar that reads: "Record: 14 of 1".

Figure 7.17. User interface for the Panel Query.

The **Panel query** of the FACS LIMS database allows the user to select subsets of flow cytometry data (cell frequency, channel statistics) and to export these with other data from the NMIC cohort. The user highlights the flow cytometry data in the tree-like structure on the left, then chooses a button (on the right) to select other patient data to add to the table (viral load, CD4, time and peak of HIV viral load, infant mortality, or delivery). Alternately, the “Query All” button adds all of these data to the output table in one step.

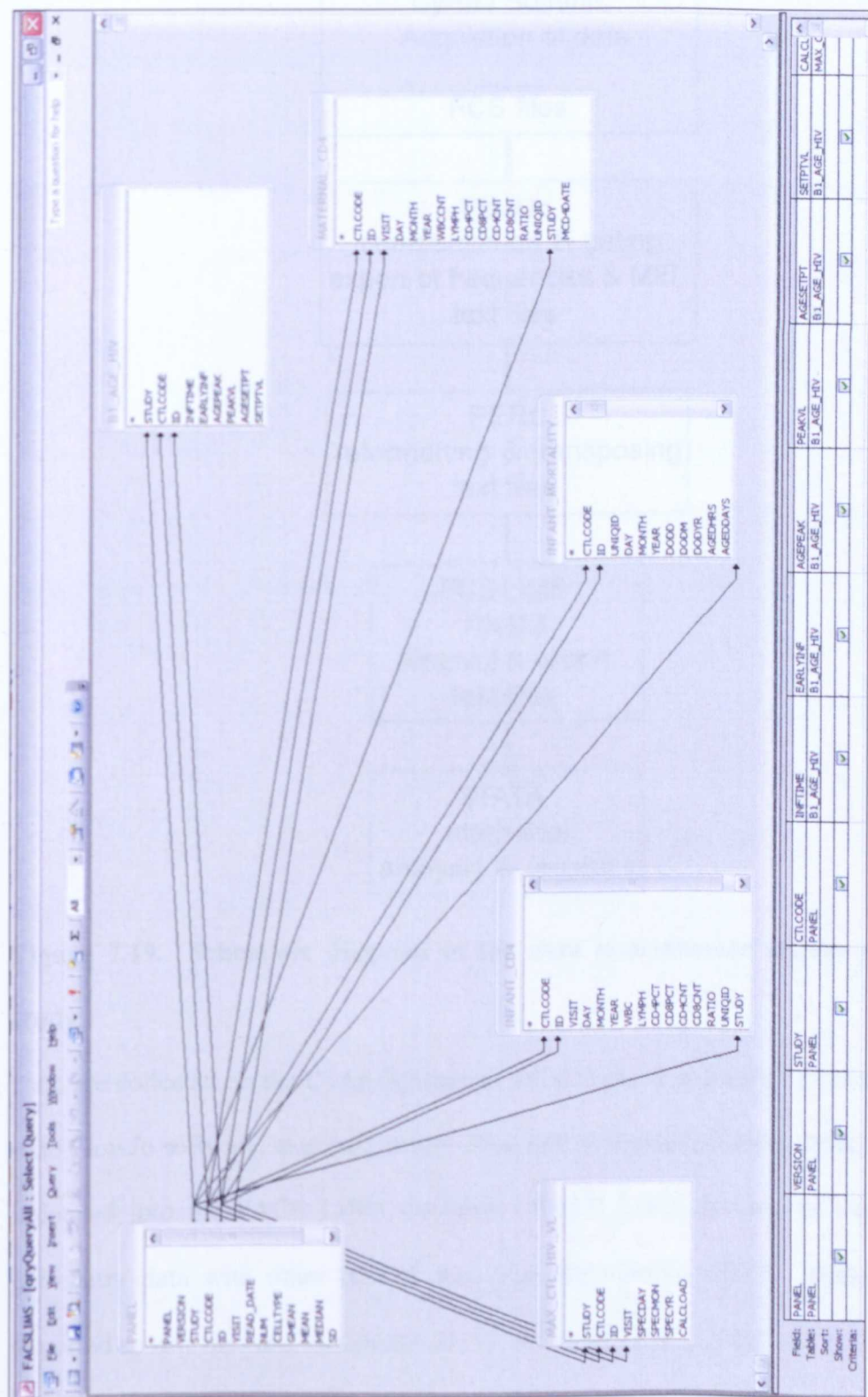


Figure 7.18. The Panel Query can be easily modified by the user to extract additional data from the database tables.

Tables are linked by key variables, allowing data to be combined from multiple tables and exported as queries.

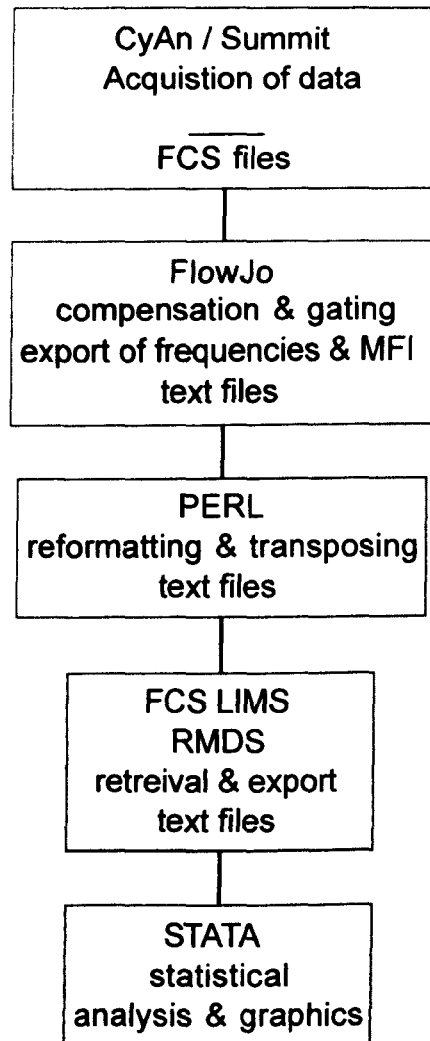


Figure 7.19. Schematic diagram of the data management system used in the study.

Data are collected on the CyAn instrument using Summit software. Files are analysed with FlowJo software, exported as text files, and re-formatted with a PERL script, and uploaded into the FACS LIMS database. FACS LIMS is used to integrate flow cytometry data with other patient data from the NMIC cohort. These tables are exported as text files and analysed with STATA statistical software.

General conclusions

The aim of this chapter was to describe some of the control experiments performed in the design and execution of the immunophenotyping experiments. Though these experiments were performed specifically to refine and understand the current study protocols, the data presented here are informative for other investigators who are setting up large cohort studies using flow cytometry data.

Before the commencement of the study, great efforts were made to monitor and control for possible sources of experimental error. Using frozen cells allowed reduction of intra-patient variability because all specimens could be tested in the same experiment. In an ideal experiment, all study specimens would be run concurrently, but the large number of specimens examined in this study made this strategy impractical. Between-subjects variability is the largest source of experimental error in this study. The establishment of a fixed healthy donor (HD) bank of cells for use in control experiments was invaluable in monitoring and controlling for the effects of time, instrument and reagent variability. Stringent quality control measures to monitor antibody stability were also an important aspect of reducing inter-assay variation and ensuring the production of usable data. Careful titration of antibodies to assure saturation of epitopes, consistency in compensation and PMT voltages were also important in limiting inter-assay error.

The upgrading of the CyAn (replacement of filters, lasers, upgrading of optics) is shown here to have significant effects upon the MFI data. These data highlight the importance of instrument variability as a source of experimental error and the need for close monitoring of this error to enable valid comparisons. Such analyses are

especially relevant in clinical trials where data may be collected over a period of months or years, during which time instruments may be serviced, upgraded or replaced several times. Ideally the same instrument would be used for the duration for the study but in practice this is not always possible. These control experiments demonstrate that a simple linear transformation may enable comparisons between different instruments.

Error introduced by manual gating remains the greatest challenge in flow cytometry studies. In this study, isotype controls were used to standardise the process and remove some of the subjectivity of the process. Auto-gating tools are useful with the analysis of some data, but are not appropriate for gating continuous, disperse, or low frequency populations. New methods of analysis are being developed which aim to eliminate the need for compensation and gating; these include cluster-analysis and principal components analysis. Data from the current cohort is being used to explore some of these novel methods, in collaboration with CBRG and Professor Marie Reilly of the Karolinska Institute in Sweden.

Investigators collecting large volumes of flow cytometry also face challenges in data storage, management and retrieval. An integrated system is outlined here that describes a streamlined process of data transfer from experiment to analysis. The FACS LIMS database is an excellent prototype for a RDMS to integrate laboratory and clinical data from large trials.

CHAPTER 8: DISCUSSION

The natural history of HIV-1 infection in Kenyan infants

Currently more than 2 million children are infected with HIV-1. Mortality is very high in HIV-1 infected children during the first two years of life in the absence of ARV therapy. In the CTL cohort, half of the children died before their second birthday despite frequent visits to clinic and close monitoring by study paediatricians. Mortality rates are likely to be even higher in rural areas of Kenya with less medical infrastructure. The pattern of viral load and CD4 during infant HIV-1 infection was strikingly different than observed in adults. The majority of infants experienced persistently high levels of viraemia throughout the observation period without reaching a “set-point” or steady state viral load. Additionally, rapid depletion of CD4 was observed, without the partial recovery observed in adults following acute infection. Opportunistic infections were common during the first few months of life, even in infants with CD4 counts above critical levels.

The current study highlights the importance of early diagnosis and treatment for HIV-1 infected infants. In adults and older children, the decision to initiate ARV therapy is made based on laboratory measurements and clinical indicators. However HIV-1 progresses so rapidly in infants that delaying the initiation of therapy could be fatal. Since this study was completed, ARV therapy has become more widely available in Kenya, but the majority of HIV-1 infected women are not accessing pMTCT interventions. There is still a great need for the expansion of voluntary counselling and testing facilities to capture more infected women before delivery, or more ideally, before they become pregnant.

Primary CMV infection: A co-factor for HIV-1 disease progression in African infants?

Co-infection with CMV has long been associated with increased mortality during AIDS in adults. Several reports also suggested an association with increased mortality in co-infected infants. The current study addressed this question by examining longitudinal CMV and HIV-1 viral loads in HIV-1 infected and exposed uninfected infants. The pattern of CMV viraemia peak and decline was delayed in HIV-1 infected infants, and many continued to be viraemic at the end of observation. Surprisingly, many of the HIV-1 exposed uninfected infants also experienced prolonged levels of plasma viraemia, suggesting that even in immunocompetent infants systemic CMV replication may not be tightly contained. A similar study should be performed in healthy HIV-1 unexposed infants to determine if systemic CMV replication observed here is a result of HIV-1 exposure.

CMV viral load has been shown to predict survival in adults with low CD4 counts or AIDS. In the current study, CMV viral load was not associated with survival in the infants when controlling for HIV-1 viral load. There may indeed be a predictive effect that the current study was underpowered to detect because of the strong effect of HIV-1 viral load. The correlation between HIV-1 and CMV viral load is likely to be explained by immunosuppression facilitating CMV replication. In the current study infants with HIV-1 who acquired CMV before 1 month of age had an increased risk of death. Interestingly, this effect was seen only in those children with relatively lower levels of HIV-1 viraemia, suggesting that CMV co-infection can significantly

increase mortality in children who would otherwise have a better prognosis. Infants co-infected with CMV at 1 month of age also had higher frequencies of activated CD4 cells. Although the link between activation and mortality cannot be made from this small study, it is plausible that the activation of CD4 T cells by CMV may contribute to HIV-1 disease. Both viruses can be transmitted to infants via the gut mucosa, and dual infection in GALT could potentially accelerate CD4 depletion.

These data illustrate the potential benefits of a neonatal CMV vaccine, and warrant further investigations into the benefits of delaying or preventing CMV infection in HIV-1 exposed neonates. Co-infection with CMV and other pathogens early in life are likely to contribute to the excess mortality observed in African infants compared to European and American cohorts.

Lymphocyte redistributions during primary viral infection

The longitudinal study of T cell phenotype during primary HIV-1 and CMV infection enabled the discrimination of virus-induced effects on lymphocyte distributions. Both HIV-1 and CMV had dramatic effects on the CD8 subset, stimulating cellular activation, apoptosis, and the development of effector and memory cells. In comparison, remarkably subtle changes were observed during primary infection in the CD4 subset. This is surprising in context of the very high levels of HIV-1 viraemia and perhaps suggests tighter homeostatic control over CD4 activation during acute infection. It is not possible to determine from the current study what proportion of these changes can be attributed to virus-specific cells.

HIV-1 specific CD8 T cells

Tetramer-staining experiments on a subset of infants demonstrated that high frequencies of HIV-1 specific CD8 T cells could be generated early in life. In one infant 3.8% of CD8 T cells were directed at a single HIV-1 epitope. Changes in the phenotype of HIV-1 specific CD8 during post-acute infection were also similar to patterns observed in adult infection. The altered expression of cellular markers examined in the current study does not explain the failure of CTL to control viraemia in infants. The dysfunction of neonatal T cells may be explained by impaired cytokine production or cytotoxicity. IFN- γ ELISpot data from this cohort revealed that HIV-1 specific CD8 responses were rare in infants at 1 month of age (~15%) but increased with age. By 3 months more than 50% of infants had a detectable response to at least one peptide and the magnitude of responses increased with age. However, neither the presence of a response, nor the magnitude or breadth of peptide-specific responses correlated with HIV-1 viral load or mortality. Together these data suggest that very young infants can indeed produce CD8 responses, but the *in vivo* priming of these responses somehow fails to generate a T cell that is able to effectively limit viral replication. Lack of CD4 help is likely to be a large component of this failure, and may also explain why CD8 IFN- γ production appears to improve with age. The ability of the infants to generate HIV-1 specific CD8 T cell responses is encouraging for the potential benefit of a CTL-based vaccine in neonates.

Concluding remarks

We offer our deepest thanks to the families who participated in these studies for the benefit of the women and children of Africa. We hope the data presented here has broadened our understanding of the processes governing HIV-1 pathogenesis in infants.

References

1. 2000. Effect of breastfeeding on infant and child mortality due to infectious diseases in less developed countries: a pooled analysis. WHO Collaborative Study Team on the Role of Breastfeeding on the Prevention of Infant Mortality. *Lancet* **355**:451-5.
2. 2006. Epidemiology of HIV/AIDS--United States, 1981-2005. *MMWR Morb Mortal Wkly Rep* **55**:589-92.
3. 1981. Kaposi's sarcoma and *Pneumocystis pneumonia* among homosexual men--New York City and California. *MMWR Morb Mortal Wkly Rep* **30**:305-8.
4. 2005. Mother-to-child transmission of HIV infection in the era of highly active antiretroviral therapy. *Clin Infect Dis* **40**:458-65.
5. 1988. Mother-to-child transmission of HIV infection. The European Collaborative Study. *Lancet* **2**:1039-43.
6. 1994. Natural history of vertically acquired human immunodeficiency virus-1 infection. The European Collaborative Study. *Pediatrics* **94**:815-9.
7. 1981. *Pneumocystis pneumonia*--Los Angeles. *MMWR Morb Mortal Wkly Rep* **30**:250-2.
8. 1982. Update on acquired immune deficiency syndrome (AIDS) among patients with hemophilia A. *MMWR Morb Mortal Wkly Rep* **31**:644-6, 652.
9. 1982. Update on acquired immune deficiency syndrome (AIDS)--United States. *MMWR Morb Mortal Wkly Rep* **31**:507-8, 513-4.
10. Addo, M. M., X. G. Yu, A. Rathod, D. Cohen, R. L. Eldridge, D. Strick, M. N. Johnston, C. Corcoran, A. G. Wurcel, C. A. Fitzpatrick, M. E. Feeney, W. R. Rodriguez, N. Basgoz, R. Draenert, D. R. Stone, C. Brander, P. J. Goulder, E. S. Rosenberg, M. Altfeld, and B. D. Walker. 2003. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J Virol* **77**:2081-92.
11. Adjorlolo-Johnson, G., K. M. De Cock, E. Ekpini, K. M. Vetter, T. Sibailly, K. Brattegaard, D. Yavo, R. Doorly, J. P. Whitaker, L. Kestens, and et al. 1994. Prospective comparison of mother-to-child transmission of HIV-1 and HIV-2 in Abidjan, Ivory Coast. *Jama* **272**:462-6.
12. Adkins, B. 1999. T-cell function in newborn mice and humans. *Immunology Today* **20**:330-335.
13. Adler, S. P. 1992. Cytomegalovirus transmission and child day care. *Adv Pediatr Infect Dis* **7**:109-22.
14. Adnan, S., A. Balamurugan, A. Trocha, M. S. Bennett, H. L. Ng, A. Ali, C. Brander, and O. O. Yang. 2006. Nef interference with HIV-1-specific CTL antiviral activity is epitope specific. *Blood* **108**:3414-9.
15. Aisen, P. 2004. Transferrin receptor 1. *Int J Biochem Cell Biol* **36**:2137-43.
16. Alce, T. M., and W. Popik. 2004. APOBEC3G is incorporated into virus-like particles by a direct interaction with HIV-1 Gag nucleocapsid protein. *J Biol Chem* **279**:34083-6.
17. Alderson, M. R., T. W. Tough, T. Davis-Smith, S. Braddy, B. Falk, K. A. Schooley, R. G. Goodwin, C. A. Smith, F. Ramsdell, and D. H. Lynch.

1995. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med* **181**:71-7.
18. **Alessio, M., S. Roggero, A. Funaro, L. B. De Monte, L. Peruzzi, M. Geuna, and F. Malavasi.** 1990. CD38 molecule: structural and biochemical analysis on human T lymphocytes, thymocytes, and plasma cells. *J Immunol* **145**:878-84.
19. **Almeida, J. R., D. A. Price, L. Papagno, Z. A. Arkoub, D. Sauce, E. Bornstein, T. E. Asher, A. Samri, A. Schnuriger, I. Theodorou, D. Costagliola, C. Rouzioux, H. Agut, A. G. Marcelin, D. Douek, B. Autran, and V. Appay.** 2007. Superior control of HIV-1 replication by CD8⁺ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* **204**:2473-85.
20. **Alt, F. W., G. D. Yancopoulos, T. K. Blackwell, C. Wood, E. Thomas, M. Boss, R. Coffman, N. Rosenberg, S. Tonegawa, and D. Baltimore.** 1984. Ordered rearrangement of immunoglobulin heavy chain variable region segments. *Embo J* **3**:1209-19.
21. **Alter, G., J. M. Malenfant, R. M. Delabre, N. C. Burgett, X. G. Yu, M. Lichterfeld, J. Zaunders, and M. Altfeld.** 2004. Increased natural killer cell activity in viremic HIV-1 infection. *J Immunol* **173**:5305-11.
22. **Altman, J., P. Moss, P. Goulder, D. Barouch, M. McHeyzer-Williams, J. Bell, A. J. McMichael, and M. Davis.** 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**:94-96.
23. **Amerongen, H. M., R. Weltzin, C. M. Farnet, P. Michetti, W. A. Haseltine, and M. R. Neutra.** 1991. Transepithelial transport of HIV-1 by intestinal M cells: a mechanism for transmission of AIDS. *J Acquir Immune Defic Syndr* **4**:760-5.
24. **Amyes, E., C. Hatton, D. Montamat-Sicotte, N. Gudgeon, A. B. Rickinson, A. J. McMichael, and M. F. Callan.** 2003. Characterization of the CD4⁺ T cell response to Epstein-Barr virus during primary and persistent infection. *J Exp Med* **198**:903-11.
25. **Anzala, A. O., T. B. Ball, T. Rostron, S. J. O'Brien, F. A. Plummer, and S. L. Rowland-Jones.** 1998. CCR2-64I allele and genotype association with delayed AIDS progression in African women. University of Nairobi Collaboration for HIV Research. *Lancet* **351**:1632-3.
26. **Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, S. Little, D. V. Havlir, D. D. Richman, N. Gruener, G. Pape, A. Waters, P. Easterbrook, M. Salio, V. Cerundolo, A. J. McMichael, and S. L. Rowland-Jones.** 2002. Memory CD8⁺ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* **8**:379-85.
27. **Appay, V., D. F. Nixon, S. M. Donahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, D. V. Havlir, D. D. Richman, A. Waters, P. Easterbrook, A. J. McMichael, and S. L. Rowland-Jones.** 2000. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J Exp Med* **192**:63-75.
28. **Appay, V., L. Papagno, C. A. Spina, P. Hansasuta, A. King, L. Jones, G. S. Ogg, S. Little, A. J. McMichael, D. D. Richman, and S. L. Rowland-Jones.** 2002. Dynamics of T cell responses in HIV infection. *J Immunol* **168**:3660-6.

29. **Appay, V., and S. L. Rowland-Jones.** 2002. The assessment of antigen-specific CD8⁺ T cells through the combination of MHC class I tetramer and intracellular staining. *J Immunol Methods* **268**:9-19.
30. **Aries, S. P., B. Schaaf, C. Muller, R. H. Dennin, and K. Dalhoff.** 1995. Fas (CD95) expression on CD4⁺ T cells from HIV-infected patients increases with disease progression. *J Mol Med* **73**:591-3.
31. **Ashorn, P., T. J. McQuade, S. Thaisrivongs, A. G. Tomasselli, W. G. Tarpley, and B. Moss.** 1990. An inhibitor of the protease blocks maturation of human and simian immunodeficiency viruses and spread of infection. *Proc Natl Acad Sci U S A* **87**:7472-6.
32. **Ausiello, C. M., A. la Sala, C. Ramoni, F. Urbani, A. Funaro, and F. Malavasi.** 1996. Secretion of IFN-gamma, IL-6, granulocyte-macrophage colony-stimulating factor and IL-10 cytokines after activation of human purified T lymphocytes upon CD38 ligation. *Cell Immunol* **173**:192-7.
33. **Backe, E., E. Jimenez, M. Unger, A. Schafer, E. Jauniaux, and M. Vogel.** 1992. Demonstration of HIV-1 infected cells in human placenta by in situ hybridisation and immunostaining. *J Clin Pathol* **45**:871-4.
34. **Balaji, K. N., N. Schaschke, W. Machleidt, M. Catalfamo, and P. A. Henkart.** 2002. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. *J Exp Med* **196**:493-503.
35. **Barbi, M., S. Binda, S. Caroppo, U. Ambrosetti, C. Corbetta, and P. Sergi.** 2003. A wider role for congenital cytomegalovirus infection in sensorineural hearing loss. *Pediatr Infect Dis J* **22**:39-42.
36. **Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dautet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**:868-71.
37. **Bartel, D. P., M. L. Zapp, M. R. Green, and J. W. Szostak.** 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. *Cell* **67**:529-36.
38. **Becker, T. C., E. J. Wherry, D. Boone, K. Murali-Krishna, R. Antia, A. Ma, and R. Ahmed.** 2002. Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J Exp Med* **195**:1541-8.
39. **Bekker, V., C. Bronke, H. J. Scherpbier, J. F. Weel, S. Jurriaans, P. M. Wertheim-van Dillen, F. van Leth, J. M. Lange, K. Tesselaar, D. van Baarle, and T. W. Kuijpers.** 2005. Cytomegalovirus rather than HIV triggers the outgrowth of effector CD8⁺CD45RA⁺CD27⁻ T cells in HIV-1-infected children. *Aids* **19**:1025-34.
40. **Bello, C., and H. Whittle.** 1991. Cytomegalovirus infection in Gambian mothers and their babies. *J Clin Pathol* **44**:366-9.
41. **Bentley, G. A., and R. A. Mariuzza.** 1996. The structure of the T cell antigen receptor. *Annu Rev Immunol* **14**:563-90.
42. **Bernard, N. F., C. M. Yannakis, J. S. Lee, and C. M. Tsoukas.** 1999. Human immunodeficiency virus (HIV)-specific cytotoxic T lymphocyte activity in HIV-exposed seronegative persons. *J Infect Dis* **179**:538-47.
43. **Bernstein, H. B., S. P. Tucker, S. R. Kar, S. A. McPherson, D. T. McPherson, J. W. Dubay, J. Lebowitz, R. W. Compans, and E. Hunter.** 1995. Oligomerization of the hydrophobic heptad repeat of gp41. *J Virol* **69**:2745-50.

44. **Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup, and L. J. Picker.** 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J Virol* **75**:11983-91.
45. **Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup.** 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* **107**:4781-9.
46. **Bevan, M. J.** 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* **143**:1283-8.
47. **Biron, K. K.** 2006. Antiviral drugs for cytomegalovirus diseases. *Antiviral Res* **71**:154-63.
48. **Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley.** 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329**:506-12.
49. **Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley.** 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* **329**:512-8.
50. **Blanche, S., M. L. Newell, M. J. Mayaux, D. T. Dunn, J. P. Teglas, C. Rouzioux, and C. S. Peckham.** 1997. Morbidity and mortality in European children vertically infected by HIV-1. The French Pediatric HIV Infection Study Group and European Collaborative Study. *J Acquir Immune Defic Syndr Hum Retrovirol* **14**:442-50.
51. **Blanden, R. V.** 1971. Mechanisms of recovery from a generalized viral infection: mousepox. 3. Regression infectious foci. *J Exp Med* **133**:1090-104.
52. **Blanden, R. V.** 1970. Mechanisms of recovery from a generalized viral infection: mousepox. I. The effects of anti-thymocyte serum. *J Exp Med* **132**:1035-54.
53. **Blanden, R. V.** 1971. Mechanisms of recovery from a generalized viral infection: mousepox. II. Passive transfer of recovery mechanisms with immune lymphoid cells. *J Exp Med* **133**:1074-89.
54. **Boasso, A., and G. M. Shearer.** 2007. Chronic innate immune activation as a cause of HIV-1 immunopathogenesis. *Clin Immunol*.
55. **Bofill, M., A. Mocroft, M. Lipman, E. Medina, N. J. Borthwick, C. A. Sabin, A. Timms, M. Winter, L. Baptista, M. A. Johnson, C. A. Lee, A. N. Phillips, and G. Janossy.** 1996. Increased numbers of primed activated CD8+CD38+CD45RO+ T cells predict the decline of CD4+ T cells in HIV-1-infected patients. *Aids* **10**:827-34.
56. **Bomse, M.** 1997. Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. *Nat Med* **3**:42-7.
57. **Boppana, S. B., R. F. Pass, W. J. Britt, S. Stagno, and C. A. Alford.** 1992. Symptomatic congenital cytomegalovirus infection: neonatal morbidity and mortality. *Pediatr Infect Dis J* **11**:93-9.
58. **Boppana, S. B., L. B. Rivera, K. B. Fowler, M. Mach, and W. J. Britt.** 2001. Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity. *N Engl J Med* **344**:1366-71.

59. **Bordet, J., and O. Bengou.** 1901. Sur l'existence de substances sensibilisatrices dans la plupart des serum antimicrobiens. *Ann Inst Pasteur* **15**:289-302.
60. **Boriskin, Y. S., M. Sharland, R. Dalton, G. duMont, and J. C. Booth.** 1999. Viral loads in dual infection with HIV-1 and cytomegalovirus. *Arch Dis Child* **80**:132-6.
61. **Borkowsky, W., M. X. Zhan, S. H. Chen, T. Ilmet, A. Kaul, S. Chandwani, M. Rigaud, S. Essajee, C. Gruber, A. Freedman, and K. Krasinski.** 2004. Correlation between HIV-Specific CD8 cell production of interferon- gamma and plasma levels of HIV RNA in perinatally infected pediatric populations. *J Infect Dis* **190**:722-6.
62. **Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone.** 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* **68**:6103-10.
63. **Bosire, R., B. Guthrie, B. Lohman-Payne, J. Mabuka, M. Majiwa, G. Wariua, D. Mbori-Ngacha, B. Richardson, G. John-Stewart, and C. Farquhar.** accepted 2007. Longitudinal comparison of chemokines in breast milk early postpartum among HIV-1-infected and uninfected Kenyan women. *Breastfeeding Medicine*.
64. **Boudet, F., H. Lecoecur, and M. L. Gougeon.** 1996. Apoptosis associated with ex vivo down-regulation of Bcl-2 and up-regulation of Fas in potential cytotoxic CD8+ T lymphocytes during HIV infection. *J Immunol* **156**:2282-93.
65. **Bowen, E. F., C. A. Sabin, P. Wilson, P. D. Griffiths, C. C. Davey, M. A. Johnson, and V. C. Emery.** 1997. Cytomegalovirus (CMV) viraemia detected by polymerase chain reaction identifies a group of HIV-positive patients at high risk of CMV disease. *Aids* **11**:889-93.
66. **Braaten, D., E. K. Franke, and J. Luban.** 1996. Cyclophilin A is required for an early step in the life cycle of human immunodeficiency virus type 1 before the initiation of reverse transcription. *J Virol* **70**:3551-60.
67. **Brahmbhatt, H., G. Kigozi, F. Wabwire-Mangen, D. Serwadda, T. Lutalo, F. Nalugoda, N. Sewankambo, M. Kiduggavu, M. Wawer, and R. Gray.** 2006. Mortality in HIV-infected and uninfected children of HIV-infected and uninfected mothers in rural Uganda. *J Acquir Immune Defic Syndr* **41**:504-8.
68. **Brenchley, J. M., N. J. Karandikar, M. R. Betts, D. R. Ambrozak, B. J. Hill, L. E. Crotty, J. P. Casazza, J. Kuruppu, S. A. Migueles, M. Connors, M. Roederer, D. C. Douek, and R. A. Koup.** 2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* **101**:2711-20.
69. **Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek.** 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* **200**:749-59.
70. **Brossard, Y., J. T. Aubin, L. Mandelbrot, C. Bignozzi, D. Brand, A. Chaput, J. Roume, N. Mulliez, F. Mallet, H. Agut, and et al.** 1995. Frequency of early in utero HIV-1 infection: a blind DNA polymerase chain reaction study on 100 fetal thymuses. *Aids* **9**:359-66.
71. **Brown, D. C., and K. C. Gatter.** 2002. Ki67 protein: the immaculate deception? *Histopathology* **40**:2-11.

72. **Browne, K. A., E. Blink, V. R. Sutton, C. J. Froelich, D. A. Jans, and J. A. Trapani.** 1999. Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. *Mol Cell Biol* **19**:8604-15.
73. **Brunner, T., R. J. Mogil, D. LaFace, N. J. Yoo, A. Mahboubi, F. Echeverri, S. J. Martin, W. R. Force, D. H. Lynch, C. F. Ware, and et al.** 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* **373**:441-4.
74. **Bukrinsky, M. I., N. Sharova, T. L. McDonald, T. Pushkarskaya, W. G. Tarpley, and M. Stevenson.** 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. *Proc Natl Acad Sci U S A* **90**:6125-9.
75. **Bunce, M., C. M. O'Neill, M. C. Barnardo, P. Krausa, M. J. Browning, P. J. Morris, and K. I. Welsh.** 1995. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5, & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens* **46**.
76. **Buseyne, F., S. Blanche, D. Schmitt, C. Griscelli, and Y. Riviere.** 1993. Detection of HIV-specific cell-mediated cytotoxicity in the peripheral blood from infected children. *J Immunol* **150**:3569-81.
77. **Buseyne, F., M. Burgard, J. P. Teglas, E. Bui, C. Rouzioux, M. J. Mayaux, S. Blanche, and Y. Riviere.** 1998. Early HIV-specific cytotoxic T lymphocytes and disease progression in children born to HIV-infected mothers. *AIDS Res Hum Retroviruses* **14**:1435-44.
78. **Buseyne, F., D. Scott-Algara, N. Bellal, M. Burgard, C. Rouzioux, S. Blanche, and Y. Riviere.** 2005. The frequency of HIV-specific interferon-gamma -producing CD8 T cells is associated with both age and level of antigenic stimulation in HIV-1-infected children. *J Infect Dis* **192**:1781-6.
79. **Buseyne, F., D. Scott-Algara, F. Porrot, B. Corre, N. Bellal, M. Burgard, C. Rouzioux, S. Blanche, and Y. Riviere.** 2002. Frequencies of ex vivo-activated human immunodeficiency virus type 1-specific gamma-interferon-producing CD8+ T cells in infected children correlate positively with plasma viral load. *J Virol* **76**:12414-22.
80. **Bushman, F. D., T. Fujiwara, and R. Craigie.** 1990. Retroviral DNA integration directed by HIV integration protein in vitro. *Science* **249**:1555-8.
81. **Callan, M. F., L. Tan, N. Annels, G. S. Ogg, J. D. Wilson, C. A. O'Callaghan, N. Steven, A. J. McMichael, and A. B. Rickinson.** 1998. Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo. *J Exp Med* **187**:1395-402.
82. **Callard, R. E., and A. J. H. Gearing.** 1994. *The Cytokine Factbook*. Academic Press, San Diego.
83. **Cameron, D. W., J. N. Simonsen, L. J. D'Costa, A. R. Ronald, G. M. Maitha, M. N. Gakinya, M. Cheang, J. O. Ndinya-Achola, P. Piot, R. C. Brunham, and et al.** 1989. Female to male transmission of human immunodeficiency virus type 1: risk factors for seroconversion in men. *Lancet* **2**:403-7.
84. **Carmichael, A., X. Jin, P. Sissons, and L. Borysiewicz.** 1993. Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection:

- differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. *J Exp Med* 177:249-56.
85. **Carrington, M., G. W. Nelson, M. P. Martin, T. Kissner, D. Vlahov, J. J. Goedert, R. Kaslow, S. Buchbinder, K. Hoots, and S. J. O'Brien.** 1999. HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science* 283:1748-52.
 86. **Carrington, M., and S. J. O'Brien.** 2003. The influence of HLA genotype on AIDS. *Annu Rev Med* 54:535-51.
 87. **Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek, and R. A. Koup.** 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. *J Exp Med* 203:2865-77.
 88. **CDC.** 2002. Guidelines for Preventing Opportunistic Infections Among HIV-Infected Persons: Recommendations of the U.S. Public Health Service and the Infectious Diseases Society of America. *MMWR* 51:1-46.
 89. **CDC.** 1998. Guidelines for the use of anti-retroviral agents in pediatric HIV infection. *MMWR*.
 90. **Chakraborty, R., A. S. Morel, J. K. Sutton, V. Appay, R. M. Ripley, T. Dong, T. Rostron, S. Ogola, T. Palakudy, R. Musoke, A. D'Agostino, M. Ritter, and S. L. Rowland-Jones.** 2005. Correlates of delayed disease progression in HIV-1-infected Kenyan children. *J Immunol* 174:8191-9.
 91. **Chan, D. C., and P. S. Kim.** 1998. HIV entry and its inhibition. *Cell* 93:681-4.
 92. **Chandwani, S., M. A. Greco, K. Mittal, C. Antoine, K. Krasinski, and W. Borkowsky.** 1991. Pathology and human immunodeficiency virus expression in placentas of seropositive women. *J Infect Dis* 163:1134-8.
 93. **Chandwani, S., A. Kaul, D. Bebenroth, M. Kim, D. D. John, A. Fidelia, A. Hassel, W. Borkowsky, and K. Krasinski.** 1996. Cytomegalovirus infection in human immunodeficiency virus type 1-infected children. *Pediatr Infect Dis J* 15:310-4.
 94. **Chen, S. F., W. W. Tu, M. A. Sharp, E. C. Tongson, X. S. He, H. B. Greenberg, T. H. Holmes, Z. Wang, G. Kemble, A. M. Manganello, S. P. Adler, C. L. Dekker, D. B. Lewis, and A. M. Arvin.** 2004. Antiviral CD8 T cells in the control of primary human cytomegalovirus infection in early childhood. *J Infect Dis* 189:1619-27.
 95. **Chen, Z., P. Telfier, A. Gettie, P. Reed, L. Zhang, D. D. Ho, and P. A. Marx.** 1996. Genetic characterization of new West African simian immunodeficiency virus SIVsm: geographic clustering of household-derived SIV strains with human immunodeficiency virus type 2 subtypes and genetically diverse viruses from a single feral sooty mangabey troop. *J Virol* 70:3617-27.
 96. **Cheynier, R., P. Langlade-Demoyen, M. R. Marescot, S. Blanche, G. Blondin, S. Wain-Hobson, C. Griscelli, E. Vilmer, and F. Plata.** 1992. Cytotoxic T lymphocyte responses in the peripheral blood of children born to human immunodeficiency virus-1-infected mothers. *Eur J Immunol* 22:2211-7.
 97. **Chiodo, F., E. Ricchi, P. Costigliola, L. Michelacci, L. Bovicelli, and P. Dallacasa.** 1986. Vertical transmission of HTLV-III. *Lancet* 1:739.

98. **Chun, T. W., D. Engel, M. M. Berrey, T. Shea, L. Corey, and A. S. Fauci.** 1998. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci U S A* **95**:8869-73.
99. **Ciechanover, A., and A. L. Schwartz.** 1994. The ubiquitin-mediated proteolytic pathway: mechanisms of recognition of the proteolytic substrate and involvement in the degradation of native cellular proteins. *Faseb J* **8**:182-91.
100. **Clavel, F., D. Guetard, F. Brun-Vezinet, S. Chamaret, M. A. Rey, M. O. Santos-Ferreira, A. G. Laurent, C. Dauguet, C. Katlama, C. Rouzioux, and et al.** 1986. Isolation of a new human retrovirus from West African patients with AIDS. *Science* **233**:343-6.
101. **Clemetson, D. B., G. B. Moss, D. M. Willerford, M. Hensel, W. Emonyi, K. K. Holmes, F. Plummer, J. Ndinya-Achola, P. L. Roberts, S. Hillier, and et al.** 1993. Detection of HIV DNA in cervical and vaginal secretions. Prevalence and correlates among women in Nairobi, Kenya. *Jama* **269**:2860-4.
102. **Clerici, M., C. Barassi, C. Devito, C. Pastori, S. Piconi, D. Trabattoni, R. Longhi, J. Hinkula, K. Broliden, and L. Lopalco.** 2002. Serum IgA of HIV-exposed uninfected individuals inhibit HIV through recognition of a region within the alpha-helix of gp41. *Aids* **16**:1731-41.
103. **Clerici, M., M. Saresella, F. Colombo, S. Fossati, N. Sala, D. Bricalli, M. L. Villa, P. Ferrante, L. Dally, and A. Vigano.** 2000. T-lymphocyte maturation abnormalities in uninfected newborns and children with vertical exposure to HIV. *Blood* **96**:3866-71.
104. **Clerici, M., M. Saresella, F. Colombo, S. Fossati, N. Sala, D. Bricalli, M. L. Villa, P. Ferrante, L. Dally, and A. Vigano.** 2000. T-lymphocyte maturation abnormalities in uninfected newborns and children with vertical exposure to HIV. *Blood* **96**:3866-71.
105. **Clerici, M., A. V. Sison, J. A. Berzofsky, T. A. Rakusan, C. D. Brandt, M. Ellaurie, M. Villa, C. Colie, D. J. Venzon, J. L. Sever, and et al.** 1993. Cellular immune factors associated with mother-to-infant transmission of HIV. *Aids* **7**:1427-33.
106. **Cocchi, F., A. L. DeVico, A. Garzino-Demo, A. Cara, R. C. Gallo, and P. Lusso.** 1996. The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. *Nat Med* **2**:1244-7.
107. **Colomb, M., and R. R. Porter.** 1975. Characterization of a plasmin-digest fragment of rabbit immunoglobulin gamma that binds antigen and complement. *Biochem J* **145**:177-83.
108. **Combadeere, B., C. Blanc, T. Li, G. Carcelain, C. Delaugerre, V. Calvez, R. Tubiana, P. Debre, C. Katlama, and B. Autran.** 2000. CD4+Ki67+ lymphocytes in HIV-infected patients are effector T cells accumulated in the G1 phase of the cell cycle. *Eur J Immunol* **30**:3598-603.
109. **Connor, E. M., R. S. Sperling, R. Gelber, P. Kiselev, G. Scott, M. J. O'Sullivan, R. VanDyke, M. Bey, W. Shearer, R. L. Jacobson, and et al.** 1994. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. *N Engl J Med* **331**:1173-80.
110. **Connor, R. I., K. E. Sheridan, D. Ceradini, S. Choe, and N. R. Landau.** 1997. Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals. *J Exp Med* **185**:621-8.

111. **Cooper, E. R., M. Charurat, L. Mofenson, I. C. Hanson, J. Pitt, C. Diaz, K. Hayani, E. Handelsman, V. Smeriglio, R. Hoff, and W. Blattner.** 2002. Combination antiretroviral strategies for the treatment of pregnant HIV-1-infected women and prevention of perinatal HIV-1 transmission. *J Acquir Immune Defic Syndr* **29**:484-94.
112. **Coovadia, H. M., N. C. Rollins, R. M. Bland, K. Little, A. Coutoudis, M. L. Bennish, and M. L. Newell.** 2007. Mother-to-child transmission of HIV-1 infection during exclusive breastfeeding in the first 6 months of life: an intervention cohort study. *Lancet* **369**:1107-16.
113. **Coutoudis, A., F. Dabis, W. Fawzi, P. Gaillard, G. Haverkamp, D. R. Harris, J. B. Jackson, V. Leroy, N. Meda, P. Msellati, M. L. Newell, R. Nsuati, J. S. Read, and S. Wiktor.** 2004. Late postnatal transmission of HIV-1 in breast-fed children: an individual patient data meta-analysis. *J Infect Dis* **189**:2154-66.
114. **Cowan, M. J., D. Hellmann, D. Chudwin, D. W. Wara, R. S. Chang, and A. J. Ammann.** 1984. Maternal transmission of acquired immune deficiency syndrome. *Pediatrics* **73**:382-6.
115. **Cresswell, P.** 1996. Invariant chain structure and MHC class II function. *Cell* **84**:505-7.
116. **Dabis, F., N. Elenga, N. Meda, V. Leroy, I. Viho, O. Manigart, L. Dequae-Merchadou, P. Msellati, and I. Sombie.** 2001. 18-Month mortality and perinatal exposure to zidovudine in West Africa. *Aids* **15**:771-9.
117. **Dao, H., L. M. Mofenson, R. Ekpini, C. F. Gilks, M. Barnhart, O. Bolu, and N. Shaffer.** 2007. International recommendations on antiretroviral drugs for treatment of HIV-infected women and prevention of mother-to-child HIV transmission in resource-limited settings: 2006 update. *Am J Obstet Gynecol* **197**:S42-55.
118. **Davis, M. M., J. J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien.** 1998. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* **16**:523-44.
119. **Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker.** 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* **443**:350-4.
120. **De Clercq, E.** 1995. Antiviral therapy for human immunodeficiency virus infections. *Clin Microbiol Rev* **8**:200-39.
121. **De Martino, M., M. E. Rossi, C. Azzari, M. G. Gelli, F. Chiarelli, L. Galli, and A. Vierucci.** 1999. Viral load and CD69 molecule expression on freshly isolated and cultured mitogen-stimulated lymphocytes of children with perinatal HIV-1 infection. *Clin Exp Immunol* **117**:513-6.
122. **de Rossi, A., L. Ometto, F. Mammano, C. Zanotto, A. del Mistro, C. Giaquinto, and L. Chieco-Bianchi.** 1993. Time course of antigenaemia and seroconversion in infants with vertically acquired HIV-1 infection. *Aids* **7**:1528-9.
123. **de Vries, E., S. de Bruin-Versteeg, W. M. Comans-Bitter, R. de Groot, W. C. Hop, G. J. Boerma, F. K. Lotgering, and J. J. van Dongen.** 2000. Longitudinal survey of lymphocyte subpopulations in the first year of life. *Pediatr Res* **47**:528-37.

124. **Deaglio, S., M. Morra, R. Mallone, C. M. Ausiello, E. Prager, G. Garbarino, U. Dianzani, H. Stockinger, and F. Malavasi.** 1998. Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. *J Immunol* **160**:395-402.
125. **Deayton, J., A. Mocroft, P. Wilson, V. C. Emery, M. A. Johnson, and P. D. Griffiths.** 1999. Loss of cytomegalovirus (CMV) viraemia following highly active antiretroviral therapy in the absence of specific anti-CMV therapy. *Aids* **13**:1203-6.
126. **Deayton, J. R., C. A. Prof Sabin, M. A. Johnson, V. C. Emery, P. Wilson, and P. D. Griffiths.** 2004. Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy. *Lancet* **363**:2116-21.
127. **Deeks, S. G., M. Smith, M. Holodniy, and J. O. Kahn.** 1997. HIV-1 protease inhibitors. A review for clinicians. *Jama* **277**:145-53.
128. **Deichmann, M., R. Kronenwett, and R. Haas.** 1997. Expression of the human immunodeficiency virus type-1 coreceptors CXCR-4 (fusin, LESTR) and CKR-5 in CD34+ hematopoietic progenitor cells. *Blood* **89**:3522-8.
129. **Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhardt, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**:661-6.
130. **Denny, T., R. Yogev, R. Gelman, C. Skuza, J. Oleske, E. Chadwick, S. C. Cheng, and E. Connor.** 1992. Lymphocyte subsets in healthy children during the first 5 years of life. *Jama* **267**:1484-8.
131. **DeVange Panteleeff, D., G. John, R. W. Nduati, D. A. Mbori-Ngacha, B. A. Richardson, J. K. Kreiss, and J. Overbaugh.** 1999. Rapid method for screening dried blood samples on filter paper for HIV type 1 DNA. *J Clin Microbiol* **37**:350-353.
132. **Devito, C., J. Hinkula, R. Kaul, J. Kimani, P. Kiama, L. Lopalco, C. Barass, S. Piconi, D. Trabattoni, J. J. Bwayo, F. Plummer, M. Clerici, and K. Broliden.** 2002. Cross-clade HIV-1-specific neutralizing IgA in mucosal and systemic compartments of HIV-1-exposed, persistently seronegative subjects. *J Acquir Immune Defic Syndr* **30**:413-20.
133. **Dhein, J., H. Walczak, C. Baumler, K. M. Debatin, and P. H. Krammer.** 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature* **373**:438-41.
134. **Diaz, C., C. Hanson, E. R. Cooper, J. S. Read, J. Watson, H. A. Mendez, J. Pitt, K. Rich, V. Smeriglio, and J. F. Lew.** 1998. Disease progression in a cohort of infants with vertically acquired HIV infection observed from birth: the Women and Infants Transmission Study (WITS). *J Acquir Immune Defic Syndr Hum Retrovirol* **18**:221-8.
135. **Dickover, R., E. Garratty, K. Yusim, C. Miller, B. Korber, and Y. Bryson.** 2006. Role of maternal autologous neutralizing antibody in selective perinatal transmission of human immunodeficiency virus type 1 escape variants. *J Virol* **80**:6525-33.
136. **Dickover, R. E., M. Dillon, K. M. Leung, P. Krogstad, S. Plaeger, S. Kwok, C. Christopherson, A. Deveikis, M. Keller, E. R. Stiehm, and Y. J. Bryson.** 1998. Early prognostic indicators in primary perinatal human immunodeficiency virus type 1 infection: importance of viral RNA and the timing of transmission on long-term outcome. *J Infect Dis* **178**:375-87.

137. **Dickover, R. E., E. M. Garratty, S. A. Herman, M. S. Sim, S. Plaeger, P. J. Boyer, M. Keller, A. Deveikis, E. R. Stiehm, and Y. J. Bryson.** 1996. Identification of levels of maternal HIV-1 RNA associated with risk of perinatal transmission. Effect of maternal zidovudine treatment on viral load. *Jama* 275:599-605.
138. **Dillner-Centerlind, M.-L., S. Hammarstrom, and P. Perlmann.** 1979. Transferrin can replace serum for in vitro growth of mitogen-stimulated T lymphocytes. *Eur J Immunol* 9:942-948.
139. **Ding, Y. H., K. J. Smith, D. N. Garboczi, U. Utz, W. E. Biddison, and D. C. Wiley.** 1998. Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. *Immunity* 8:403-11.
140. **Diosi, P., L. Babusceac, O. Nevinglovschi, and G. Kun-Stoicu.** 1967. Cytomegalovirus infection associated with pregnancy. *Lancet* 2:1063-6.
141. **Dobmeyer, T. S., S. A. Klein, J. M. Dobmeyer, B. Raffel, S. Findhammer, D. Hoelzer, E. B. Helm, R. Rossol, and D. Kabelitz.** 1998. Differential expression of bcl-2 and susceptibility to programmed cell death in lymphocytes of HIV-1-infected individuals. *Clin Immunol Immunopathol* 87:230-9.
142. **Dockrell, D. H., A. D. Badley, A. Algeciras-Schimnich, M. Simpson, R. Schut, D. H. Lynch, and C. V. Paya.** 1999. Activation-induced CD4+ T cell death in HIV-positive individuals correlates with Fas susceptibility, CD4+ T cell count, and HIV plasma viral copy number. *AIDS Res Hum Retroviruses* 15:1509-18.
143. **Dorrell, L.** 2005. Therapeutic immunization strategies for the control of HIV-1. *Expert Rev Vaccines* 4:513-20.
144. **Dorrell, L., H. Yang, B. Ondondo, T. Dong, K. di Gleria, A. Suttill, C. Conlon, D. Brown, P. Williams, P. Bowness, N. Goonetilleke, T. Rostron, S. Rowland-Jones, T. Hanke, and A. McMichael.** 2006. Expansion and diversification of virus-specific T cells following immunization of human immunodeficiency virus type 1 (HIV-1)-infected individuals with a recombinant modified vaccinia virus Ankara/HIV-1 Gag vaccine. *J Virol* 80:4705-16.
145. **Douek, D. C., J. M. Brenchley, M. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. Price, M. Connors, and R. Koup.** 2002. HIV preferentially infects HIV-specific CD4+ T cells. *Nature* 417:95-98.
146. **Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup.** 2002. HIV preferentially infects HIV-specific CD4+ T cells. *Nature* 417:95-8.
147. **Douek, D. C., and R. A. Koup.** 2000. Evidence for thymic function in the elderly. *Vaccine* 18:1638-41.
148. **Douek, D. C., R. D. McFarland, P. H. Keiser, E. A. Gage, J. M. Massey, B. F. Haynes, M. A. Polis, A. T. Haase, M. B. Feinberg, J. L. Sullivan, B. D. Jamieson, J. A. Zack, L. J. Picker, and R. A. Koup.** 1998. Changes in thymic function with age and during the treatment of HIV infection. *Nature* 396:690-5.

149. **Doyle, M., J. T. Atkins, and I. R. Rivera-Matos.** 1996. Congenital cytomegalovirus infection in infants infected with human immunodeficiency virus type 1. *Pediatr Infect Dis J* **15**:1102-6.
150. **Draenert, R., S. Le Gall, K. J. Pfafferott, A. J. Leslie, P. Chetty, C. Brander, E. C. Holmes, S. C. Chang, M. E. Feeney, M. M. Addo, L. Ruiz, D. Ramduth, P. Jeena, M. Altfeld, S. Thomas, Y. Tang, C. L. Verrill, C. Dixon, J. G. Prado, P. Kiepiela, J. Martinez-Picado, B. D. Walker, and P. J. Goulder.** 2004. Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1 infection. *J Exp Med* **199**:905-15.
151. **Drew, W. L.** 1988. Cytomegalovirus infection in patients with AIDS. *J Infect Dis* **158**:449-56.
152. **Dreyer, W. J., and J. C. Bennett.** 1965. The molecular basis of antibody formation: a paradox. *Proc Natl Acad Sci U S A* **54**:864-9.
153. **Duvall, M. G., A. Jaye, T. Dong, J. M. Brenchley, A. S. Alabi, D. J. Jeffries, M. van der Sande, T. O. Togun, S. J. McConkey, D. C. Douek, A. J. McMichael, H. C. Whittle, R. A. Koup, and S. L. Rowland-Jones.** 2006. Maintenance of HIV-specific CD4⁺ T cell help distinguishes HIV-2 from HIV-1 infection. *J Immunol* **176**:6973-81.
154. **Edwards, B. H., A. Bansal, S. Sabbaj, J. Bakari, M. J. Mulligan, and P. A. Goepfert.** 2002. Magnitude of functional CD8⁺ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. *J Virol* **76**:2298-305.
155. **Ehrnst, A., S. Lindgren, M. Dictor, B. Johansson, A. Sonnerborg, J. Czajkowski, G. Sundin, and A. B. Bohlin.** 1991. HIV in pregnant women and their offspring: evidence for late transmission. *Lancet* **338**:203-7.
156. **Embree, J., J. Bwayo, N. Nagelkerke, S. Njenga, P. Nyange, J. Ndinya-Achola, H. Pamba, and F. Plummer.** 2001. Lymphocyte subsets in human immunodeficiency virus type 1-infected and uninfected children in Nairobi. *Pediatr Infect Dis J* **20**:397-403.
157. **Emery, S., S. Bodrug, B. A. Richardson, C. Giachetti, M. A. Bott, D. Panteleeff, L. L. Jagodzinski, N. L. Michael, R. Nduati, J. Bwayo, J. K. Kreiss, and J. Overbaugh.** 2000. Evaluation of performance of the Gen-Probe human immunodeficiency virus type 1 viral load assay using primary subtype A, C, and D isolates from Kenya. *J Clin Microbiol* **38**:2688-95.
158. **Emery, V. C.** 2001. Investigation of CMV disease in immunocompromised patients. *J Clin Pathol* **54**:84-8.
159. **Emery, V. C., C. Sabin, J. E. Feinberg, M. Grywacz, S. Knight, and P. D. Griffiths.** 1999. Quantitative effects of valgacyclovir on the replication of cytomegalovirus (CMV) in persons with advanced human immunodeficiency virus disease: baseline CMV load dictates time to disease and survival. The AIDS Clinical Trials Group 204/Glaxo Wellcome 123-014 International CMV Prophylaxis Study Group. *J Infect Dis* **180**:695-701.
160. **Eshleman, S. H., M. J. Gonzales, G. Becker-Pergola, S. C. Cunningham, L. A. Guay, J. B. Jackson, and R. W. Shafer.** 2002. Identification of Ugandan HIV type 1 variants with unique patterns of recombination in pol involving subtypes A and D. *AIDS Res Hum Retroviruses* **18**:507-11.
161. **Evans, T. G., E. G. Kallas, A. E. Luque, M. Menegus, C. McNair, and R. J. Looney.** 1999. Expansion of the CD57 subset of CD8 T cells in HIV-1 infection is related to CMV serostatus. *Aids* **13**:1139-41.

162. **Fahey, J. L., J. M. Taylor, R. Detels, B. Hofmann, R. Melmed, P. Nishanian, and J. V. Giorgi.** 1990. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. *N Engl J Med* **322**:166-72.
163. **Farquhar, C., D. A. Mbori-Ngacha, M. W. Redman, R. K. Bosire, B. L. Lohman, A. L. Piantadosi, R. B. Goodman, J. T. Ruzinski, S. R. Emery, C. H. Crudder, J. M. Overbaugh, and G. C. John-Stewart.** 2005. CC and CXC chemokines in breastmilk are associated with mother-to-child HIV-1 transmission. *Curr HIV Res* **3**:361-9.
164. **Farquhar, C., S. Rowland-Jones, D. Mbori-Ngacha, M. Redman, B. Lohman, J. Slyker, P. Otieno, E. Obimbo, T. Rostron, J. Ochieng, J. Oyugi, R. Bosire, and G. John-Stewart.** 2004. Human leukocyte antigen (HLA) B*18 and protection against mother-to-child HIV type 1 transmission. *AIDS Res Hum Retroviruses* **20**:692-7.
165. **Farquhar, C., T. C. VanCott, D. A. Mbori-Ngacha, L. Horani, R. K. Bosire, J. K. Kreiss, B. A. Richardson, and G. C. John-Stewart.** 2002. Salivary secretory leukocyte protease inhibitor is associated with reduced transmission of human immunodeficiency virus type 1 through breast milk. *J Infect Dis* **186**:1173-6.
166. **Fassinou, P., N. Elenga, F. Rouet, R. Laguide, K. A. Kouakoussui, M. Timite, S. Blanche, and P. Msellati.** 2004. Highly active antiretroviral therapies among HIV-1-infected children in Abidjan, Cote d'Ivoire. *Aids* **18**:1905-13.
167. **Fauci, A. S., S. M. Schnittman, G. Poli, S. Koenig, and G. Pantaleo.** 1991. NIH conference. Immunopathogenic mechanisms in human immunodeficiency virus (HIV) infection. *Ann Intern Med* **114**:678-93.
168. **Fawzi, W. W., R. L. Mbise, E. Hertzmark, M. R. Fataki, M. G. Herrera, G. Ndossi, and D. Spiegelman.** 1999. A randomized trial of vitamin A supplements in relation to mortality among human immunodeficiency virus-infected and uninfected children in Tanzania. *Pediatr Infect Dis J* **18**:127-33.
169. **Faye, A., J. Le Chenadec, C. Dollfus, I. Thuret, D. Douard, G. Firtion, E. Lachassinne, M. Levine, J. Nicolas, F. Monpoux, J. Tricoire, C. Rouzioux, M. Tardieu, M. J. Mayaux, and S. Blanche.** 2004. Early versus deferred antiretroviral multidrug therapy in infants infected with HIV type 1. *Clin Infect Dis* **39**:1692-8.
170. **Feeney, M. E., Y. Tang, K. Pfafferott, K. A. Roosevelt, R. Draenert, A. Trocha, X. G. Yu, C. Verrill, T. Allen, C. Moore, S. Mallal, S. Burchett, K. McIntosh, S. I. Pelton, M. A. St John, R. Hazra, P. Klenerman, M. Altfeld, B. D. Walker, and P. J. Goulder.** 2005. HIV-1 viral escape in infancy followed by emergence of a variant-specific CTL response. *J Immunol* **174**:7524-30.
171. **Feinberg, M. B., D. Baltimore, and A. D. Frankel.** 1991. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc Natl Acad Sci U S A* **88**:4045-9.
172. **Feng, S., and E. C. Holland.** 1988. HIV-1 tat trans-activation requires the loop sequence within tar. *Nature* **334**:165-7.
173. **Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**:872-7.

174. **Feske, S., J. Giltzane, R. Dolmetsch, L. M. Staudt, and A. Rao.** 2001. Gene regulation mediated by calcium signals in T lymphocytes. *Nat Immunol* 2:316-24.
175. **Fiala, M., J. E. Payne, T. V. Berne, T. C. Moore, W. Henle, J. Z. Montgomerie, S. N. Chatterjee, and L. B. Guze.** 1975. Epidemiology of cytomegalovirus infection after transplantation and immunosuppression. *J Infect Dis* 132:421-33.
176. **Fideli, U. S., S. A. Allen, R. Musonda, S. Trask, B. H. Hahn, H. Weiss, J. Mulenga, F. Kasolo, S. H. Vermund, and G. M. Aldrovandi.** 2001. Virologic and immunologic determinants of heterosexual transmission of human immunodeficiency virus type 1 in Africa. *AIDS Res Hum Retroviruses* 17:901-10.
177. **Finkel, T. H., G. Tudor-Williams, N. K. Banda, M. F. Cotton, T. Curiel, C. Monks, T. W. Baba, R. M. Ruprecht, and A. Kupfer.** 1995. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nat Med* 1:129-34.
178. **Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano.** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* 278:1295-300.
179. **Flexner, C.** 1998. HIV-protease inhibitors. *N Engl J Med* 338:1281-92.
180. **Flores-Villanueva, P. O., E. J. Yunis, J. C. Delgado, E. Vittinghoff, S. Buchbinder, J. Y. Leung, A. M. Ugialoro, O. P. Clavijo, E. S. Rosenberg, S. A. Kalams, J. D. Braun, S. L. Boswell, B. D. Walker, and A. E. Goldfeld.** 2001. Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. *Proc Natl Acad Sci U S A* 98:5140-5.
181. **Flynn, N. M., D. N. Forthal, C. D. Harro, F. N. Judson, K. H. Mayer, and M. F. Para.** 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis* 191:654-65.
182. **Forrest, D. M., E. Seminari, R. S. Hogg, B. Yip, J. Raboud, L. Lawson, P. Phillips, M. T. Schechter, M. V. O'Shaughnessy, and J. S. Montaner.** 1998. The incidence and spectrum of AIDS-defining illnesses in persons treated with antiretroviral drugs. *Clin Infect Dis* 27:1379-85.
183. **Fowke, K. R., R. Kaul, K. L. Rosenthal, J. Oyugi, J. Kimani, W. J. Rutherford, N. J. Nagelkerke, T. B. Ball, J. J. Bwayo, J. N. Simonsen, G. M. Shearer, and F. A. Plummer.** 2000. HIV-1-specific cellular immune responses among HIV-1-resistant sex workers. *Immunol Cell Biol* 78:586-95.
184. **Fowke, K. R., N. J. Nagelkerke, J. Kimani, J. N. Simonsen, A. O. Anzala, J. J. Bwayo, K. S. MacDonald, E. N. Ngugi, and F. A. Plummer.** 1996. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. *Lancet* 348:1347-51.
185. **Fowler, K. B., S. Stagno, and R. F. Pass.** 2003. Maternal immunity and prevention of congenital cytomegalovirus infection. *Jama* 289:1008-11.
186. **Fowler, K. B., S. Stagno, R. F. Pass, W. J. Britt, T. J. Boll, and C. A. Alford.** 1992. The outcome of congenital cytomegalovirus infection in relation to maternal antibody status. *N Engl J Med* 326:663-7.
187. **Frankel, S. S., K. Tenner-Racz, P. Racz, B. M. Wenig, C. H. Hansen, D. Heffner, A. M. Nelson, M. Pope, and R. M. Steinman.** 1997. Active

- replication of HIV-1 at the lymphoepithelial surface of the tonsil. *Am J Pathol* **151**:89-96.
188. **Frankel, S. S., B. M. Wenig, A. P. Burke, P. Mannan, L. D. Thompson, S. L. Abbondanzo, A. M. Nelson, M. Pope, and R. M. Steinman.** 1996. Replication of HIV-1 in dendritic cell-derived syncytia at the mucosal surface of the adenoid. *Science* **272**:115-7.
 189. **Fraser, S. A., R. Karimi, M. Michalak, and D. Hudig.** 2000. Perforin lytic activity is controlled by calreticulin. *J Immunol* **164**:4150-5.
 190. **Froebel, K. S., G. M. Raab, C. D'Alessandro, M. P. Armitage, K. M. MacKenzie, M. Struthers, J. M. Whitelaw, and S. Yang.** 2000. A single measurement of CD38CD8 cells in HIV+, long-term surviving injecting drug users distinguishes those who will progress to AIDS from those who will remain stable. *Clin Exp Immunol* **122**:72-8.
 191. **Fujita, T., M. Matsushita, and Y. Endo.** 2004. The lectin-complement pathway--its role in innate immunity and evolution. *Immunol Rev* **198**:185-202.
 192. **Gallay, P., S. Swingler, C. Aiken, and D. Trono.** 1995. HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *Cell* **80**:379-88.
 193. **Gallay, P., S. Swingler, J. Song, F. Bushman, and D. Trono.** 1995. HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* **83**:569-76.
 194. **Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, and et al.** 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**:500-3.
 195. **Gandhi, M. K., and R. Khanna.** 2004. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect Dis* **4**:725-38.
 196. **Gao, F., E. Bailes, D. L. Robertson, Y. Chen, C. M. Rodenburg, S. F. Michael, L. B. Cummins, L. O. Arthur, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 1999. Origin of HIV-1 in the chimpanzee *Pan troglodytes*. *Nature* **397**:436-41.
 197. **Garcia, J. V., and A. D. Miller.** 1992. Downregulation of cell surface CD4 by nef. *Res Virol* **143**:52-5.
 198. **Garcia, K. C., M. Degano, R. L. Stanfield, A. Brunmark, M. R. Jackson, P. A. Peterson, L. Teyton, and I. A. Wilson.** 1996. An alphabeta T cell receptor structure at 2.5 Å and its orientation in the TCR-MHC complex. *Science* **274**:209-19.
 199. **Gavin, M. A., T. R. Torgerson, E. Houston, P. DeRoos, W. Y. Ho, A. Stray-Pedersen, E. L. Ocheltree, P. D. Greenberg, H. D. Ochs, and A. Y. Rudensky.** 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* **103**:6659-64.
 200. **Gaytant, M. A., J. M. Galama, B. A. Semmekrot, W. J. Melchers, J. M. Sporken, H. P. Oosterbaan, P. A. van Dop, A. Huisman, H. M. Merkus, and E. A. Steegers.** 2005. The incidence of congenital cytomegalovirus infections in The Netherlands. *J Med Virol* **76**:71-5.
 201. **Gea-Banacloche, J. C., S. A. Migueles, L. Martino, W. L. Shupert, A. C. McNeil, M. S. Sabbaghian, L. Ehler, C. Prussin, R. Stevens, L. Lambert,**

- J. Altman, C. W. Hallahan, J. C. de Quiros, and M. Connors. 2000. Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors. *J Immunol* **165**:1082-92.
202. Geijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. KewalRamani, D. R. Littman, C. G. Figdor, and Y. van Kooyk. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**:587-97.
203. Gerdes, J., H. Lemke, H. Baisch, H. H. Wacker, U. Schwab, and H. Stein. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* **133**:1710-5.
204. Gerdes, J., U. Schwab, H. Lemke, and H. Stein. 1983. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer* **31**:13-20.
205. Gichuhi, C., E. Obimbo, D. Mbori-Ngacha, A. Mwatha, P. Otieno, C. Farquhar, G. Wariua, D. Wamalwa, R. Bosire, and G. John-Stewart. 2005. Predictors of mortality in HIV-1 exposed uninfected post-neonatal infants at the Kenyatta National Hospital, Nairobi. *East Afr Med J* **82**:447-51.
206. Gillespie, G. M., M. R. Wills, V. Appay, C. O'Callaghan, M. Murphy, N. Smith, P. Sissons, S. Rowland-Jones, J. I. Bell, and P. A. Moss. 2000. Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. *J Virol* **74**:8140-50.
207. Giorgi, J. V., L. E. Hultin, J. A. McKeating, T. D. Johnson, B. Owens, L. P. Jacobson, R. Shih, J. Lewis, D. J. Wiley, J. P. Phair, S. M. Wolinsky, and R. Detels. 1999. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis* **179**:859-70.
208. Goh, W. C., J. Markee, R. E. Akridge, M. Meldorf, L. Musey, T. Karchmer, M. Krone, A. Collier, L. Corey, M. Emerman, and M. J. McElrath. 1999. Protection against human immunodeficiency virus type 1 infection in persons with repeated exposure: evidence for T cell immunity in the absence of inherited CCR5 coreceptor defects. *J Infect Dis* **179**:548-57.
209. Gomez, C., and T. J. Hope. 2005. The ins and outs of HIV replication. *Cell Microbiol* **7**:621-6.
210. Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, K. Raphael, and et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**:676-82.
211. Gotch, F. M., D. F. Nixon, N. Alp, A. J. McMichael, and L. K. Borysiewicz. 1990. High frequency of memory and effector gag specific cytotoxic T lymphocytes in HIV seropositive individuals. *Int Immunol* **2**:707-12.
212. Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine. 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **86**:5781-5.
213. Goudsmit, J., C. Debouck, R. H. Meloen, L. Smit, M. Bakker, D. M. Asher, A. V. Wolff, C. J. Gibbs, Jr., and D. C. Gajdusek. 1988. Human

- immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. *Proc Natl Acad Sci U S A* **85**:4478-82.
214. **Goulder, P. J., C. Brander, Y. Tang, C. Tremblay, R. A. Colbert, M. M. Addo, E. S. Rosenberg, T. Nguyen, R. Allen, A. Trocha, M. Altfeld, S. He, M. Bunce, R. Funkhouser, S. I. Pelton, S. K. Burchett, K. McIntosh, B. T. Korber, and B. D. Walker.** 2001. Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature* **412**:334-8.
 215. **Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones.** 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* **3**:212-7.
 216. **Gray, L., M. L. Newell, C. Thorne, C. Peckham, and J. Levy.** 2001. Fluctuations in symptoms in human immunodeficiency virus-infected children: the first 10 years of life. *Pediatrics* **108**:116-22.
 217. **Gray, R. H., M. J. Wawer, R. Brookmeyer, N. K. Sewankambo, D. Serwadda, F. Wabwire-Mangen, T. Lutalo, X. Li, T. vanCott, and T. C. Quinn.** 2001. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *Lancet* **357**:1149-53.
 218. **Griffiths, P. D.** 2006. CMV as a cofactor enhancing progression of AIDS. *J Clin Virol* **35**:489-92.
 219. **Grossman, Z., M. Meier-Schellersheim, W. E. Paul, and L. J. Picker.** 2006. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nat Med* **12**:289-95.
 220. **Guadalupe, M., E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. McNeil, and S. Dandekar.** 2003. Severe CD4⁺ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* **77**:11708-17.
 221. **Guermonprez, P., and S. Amigorena.** 2005. Pathways for antigen cross presentation. *Springer Semin Immunopathol* **26**:257-71.
 222. **Gupta, A., U. Nayak, M. Ram, R. Bhosale, S. Patil, A. Basavraj, A. Kakrani, S. Philip, D. Desai, J. Sastry, and R. C. Bollinger.** 2007. Postpartum tuberculosis incidence and mortality among HIV-infected women and their infants in Pune, India, 2002-2005. *Clin Infect Dis* **45**:241-9.
 223. **Guthrie, B. L., G. de Bruyn, and C. Farquhar.** 2007. HIV-1-discordant couples in sub-Saharan Africa: explanations and implications for high rates of discordancy. *Curr HIV Res* **5**:416-29.
 224. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and transactivation of the human immunodeficiency virus type 2. *Nature* **326**:662-9.
 225. **Hahn, B. H., G. M. Shaw, K. M. De Cock, and P. M. Sharp.** 2000. AIDS as a zoonosis: scientific and public health implications. *Science* **287**:607-14.
 226. **Haigwood, N. L., A. Watson, W. F. Sutton, J. McClure, A. Lewis, J. Ranchalis, B. Travis, G. Voss, N. L. Letvin, S. L. Hu, V. M. Hirsch, and P. R. Johnson.** 1996. Passive immune globulin therapy in the SIV/macaque model: early intervention can alter disease profile. *Immunol Lett* **51**:107-14.

227. **Hamprecht, K., J. Maschmann, M. Vochem, K. Dietz, C. P. Speer, and G. Jahn.** 2001. Epidemiology of transmission of cytomegalovirus from mother to preterm infant by breastfeeding. *Lancet* **357**:513-8.
228. **Hansasuta, P., T. Dong, H. Thananchai, M. Weekes, C. Willberg, H. Aldemir, S. Rowland-Jones, and V. M. Braud.** 2004. Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol* **34**:1673-9.
229. **Harari, A., S. Petitpierre, F. Vallelleian, and G. Pantaleo.** 2004. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood* **103**:966-72.
230. **Harding, R., and A. D. Bocking.** 2001. Fetal growth and development. Cambridge University Press, Cambridge.
231. **Haridas, V., T. W. McCloskey, R. Pahwa, and S. Pahwa.** 2003. Discordant expression of perforin and granzyme A in total and HIV-specific CD8 T lymphocytes of HIV infected children and adolescents. *Aids* **17**:2313-22.
232. **Harrington, L. E., P. R. Mangan, and C. T. Weaver.** 2006. Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Curr Opin Immunol* **18**:349-56.
233. **Harris, R. S., S. K. Petersen-Mahrt, and M. S. Neuberger.** 2002. RNA editing enzyme APOBEC1 and some of its homologs can act as DNA mutators. *Mol Cell* **10**:1247-53.
234. **Hassan-Walker, A. F., F. M. Mattes, P. D. Griffiths, and V. C. Emery.** 2001. Quantity of cytomegalovirus DNA in different leukocyte populations during active infection in vivo and the presence of gB and UL18 transcripts. *J Med Virol* **64**:283-9.
235. **Hellerstein, M. K., and J. M. McCune.** 1997. T cell turnover in HIV-1 disease. *Immunity* **7**:583-9.
236. **Hendel, H., S. Caillat-Zucman, H. Lebuanec, M. Carrington, S. O'Brien, J. M. Andrieu, F. Schachter, D. Zagury, J. Rappaport, C. Winkler, G. W. Nelson, and J. F. Zagury.** 1999. New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS. *J Immunol* **162**:6942-6.
237. **Hendriks, J., L. A. Gravestein, K. Tesselaar, R. A. van Lier, T. N. Schumacher, and J. Borst.** 2000. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol* **1**:433-40.
238. **Hendriks, J., Y. Xiao, and J. Borst.** 2003. CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J Exp Med* **198**:1369-80.
239. **Hertz, M., and D. Nemazee.** 1997. BCR ligation induces receptor editing in IgM+IgD- bone marrow B cells in vitro. *Immunity* **6**:429-36.
240. **Hildreth, J. E., and R. J. Orentas.** 1989. Involvement of a leukocyte adhesion receptor (LFA-1) in HIV-induced syncytium formation. *Science* **244**:1075-8.
241. **Ho, H. N., L. E. Hultin, R. T. Mitsuyasu, J. L. Matud, M. A. Hausner, D. Bockstoe, C. C. Chou, S. O'Rourke, J. M. Taylor, and J. V. Giorgi.** 1993. Circulating HIV-specific CD8+ cytotoxic T cells express CD38 and HLA-DR antigens. *J Immunol* **150**:3070-9.
242. **Hockenbery, D., G. Nunez, C. Milliman, R. D. Schreiber, and S. J. Korsmeyer.** 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**:334-6.

243. **Howarth, M., A. Williams, A. B. Tolstrup, and T. Elliott.** 2004. Tapasin enhances MHC class I peptide presentation according to peptide half-life. *Proc Natl Acad Sci U S A* **101**:11737-42.
244. **Huppa, J. B., and M. M. Davis.** 2003. T-cell-antigen recognition and the immunological synapse. *Nat Rev Immunol* **3**:973-83.
245. **Hwang, S. S., T. J. Boyle, H. K. Lyerly, and B. R. Cullen.** 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* **253**:71-4.
246. **Hyjek, E., H. W. Lischner, T. Hyslop, J. Bartkowiak, M. Kubin, G. Trinchieri, and D. Kozbor.** 1995. Cytokine patterns during progression to AIDS in children with perinatal HIV infection. *J Immunol* **155**:4060-71.
247. **Iliff, P. J., E. G. Piwoz, N. V. Tavengwa, C. D. Zunguza, E. T. Marinda, K. J. Nathoo, L. H. Moulton, B. J. Ward, and J. H. Humphrey.** 2005. Early exclusive breastfeeding reduces the risk of postnatal HIV-1 transmission and increases HIV-free survival. *Aids* **19**:699-708.
248. **Im, E. J., N. Saubi, G. Virgili, C. Sander, D. Teoh, J. M. Gatell, H. McShane, J. Joseph, and T. Hanke.** 2007. Vaccine platform for prevention of tuberculosis and mother-to-child transmission of human immunodeficiency virus type 1 through breastfeeding. *J Virol* **81**:9408-18.
249. **Ioannidis, J. P., A. Tatsioni, E. J. Abrams, M. Bulterys, R. W. Coombs, J. J. Goedert, B. T. Korber, M. J. Mayaux, L. M. Mofenson, J. Moye, Jr., M. L. Newell, D. E. Shapiro, J. P. Teglas, B. Thompson, and J. Wiener.** 2004. Maternal viral load and rate of disease progression among vertically HIV-1-infected children: an international meta-analysis. *Aids* **18**:99-108.
250. **Iversen, A. K., G. Stewart-Jones, G. H. Learn, N. Christie, C. Sylvester-Hviid, A. E. Armitage, R. Kaul, T. Beattie, J. K. Lee, Y. Li, P. Chotiyarnwong, T. Dong, X. Xu, M. A. Luscher, K. MacDonald, H. Ullum, B. Klarlund-Pedersen, P. Skinhoj, L. Fugger, S. Buus, J. I. Mullins, E. Y. Jones, P. A. van der Merwe, and A. J. McMichael.** 2006. Conflicting selective forces affect T cell receptor contacts in an immunodominant human immunodeficiency virus epitope. *Nat Immunol* **7**:179-89.
251. **Iwai, K., T. Miyawaki, T. Takizawa, A. Konno, K. Ohta, A. Yachie, H. Seki, and N. Taniguchi.** 1994. Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. *Blood* **84**:1201-8.
252. **Iwata, M., Y. Ohoka, T. Kuwata, and A. Asada.** 1996. Regulation of T cell apoptosis via T cell receptors and steroid receptors. *Stem Cells* **14**:632-41.
253. **Jacks, T., M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus.** 1988. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. *Nature* **331**:280-3.
254. **Jacobson, M. A., and J. Mills.** 1988. Serious cytomegalovirus disease in the acquired immunodeficiency syndrome (AIDS). Clinical findings, diagnosis, and treatment. *Ann Intern Med* **108**:585-94.
255. **Jacobson, M. A., H. Stanley, C. Holtzer, T. P. Margolis, and E. T. Cunningham.** 2000. Natural history and outcome of new AIDS-related cytomegalovirus retinitis diagnosed in the era of highly active antiretroviral therapy. *Clin Infect Dis* **30**:231-3.

256. **Jaffar, S., A. D. Grant, J. Whitworth, P. G. Smith, and H. Whittle.** 2004. The natural history of HIV-1 and HIV-2 infections in adults in Africa: a literature review. *Bull World Health Organ* **82**:462-9.
257. **Jandl, J. H., J. K. Inman, R. L. Simmons, and D. W. Allen.** 1959. Transfer of iron from serum iron-binding protein to human reticulocytes. *Journal of Clinical Investigation* **38**:161-185.
258. **Janini, M., M. Rogers, D. R. Birx, and F. E. McCutchan.** 2001. Human immunodeficiency virus type 1 DNA sequences genetically damaged by hypermutation are often abundant in patient peripheral blood mononuclear cells and may be generated during near-simultaneous infection and activation of CD4(+) T cells. *J Virol* **75**:7973-86.
259. **Jenkins, M., D. Landers, D. Williams-Herman, D. Wara, R. R. Viscarello, H. A. Hammill, M. W. Kline, W. T. Shearer, E. D. Charlebois, and S. Kohl.** 1994. Association between anti-human immunodeficiency virus type 1 (HIV-1) antibody-dependent cellular cytotoxicity antibody titers at birth and vertical transmission of HIV-1. *J Infect Dis* **170**:308-12.
260. **Jennings, C., K. Rich, J. N. Siegel, and A. Landay.** 1994. A phenotypic study of CD8+ lymphocyte subsets in infants using three-color flow cytometry. *Clin Immunol Immunopathol* **71**:8-13.
261. **Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho.** 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* **189**:991-8.
262. **John, G. C., T. Bird, J. Overbaugh, R. Nduati, D. Mbori-Ngacha, T. Rostron, T. Dong, L. Kostrikis, B. Richardson, and S. L. Rowland-Jones.** 2001. CCR5 promoter polymorphisms in a Kenyan perinatal human immunodeficiency virus type 1 cohort: association with increased 2-year maternal mortality. *J Infect Dis* **184**:89-92.
263. **John, G. C., R. W. Nduati, D. A. Mbori-Ngacha, B. A. Richardson, D. Panteleeff, A. Mwatha, J. Overbaugh, J. Bwayo, J. O. Ndinya-Achola, and J. K. Kreiss.** 2001. Correlates of mother-to-child human immunodeficiency virus type 1 (HIV-1) transmission: association with maternal plasma HIV-1 RNA load, genital HIV-1 DNA shedding, and breast infections. *J Infect Dis* **183**:206-212.
264. **John, G. C., C. Rousseau, T. Dong, S. Rowland-Jones, R. Nduati, D. Mbori-Ngacha, T. Rostron, J. K. Kreiss, B. A. Richardson, and J. Overbaugh.** 2000. Maternal SDF1 3'A polymorphism is associated with increased perinatal human immunodeficiency virus type 1 transmission. *J Virol* **74**:5736-9.
265. **Jonjic, S., W. Mutter, F. Weiland, M. J. Reddehase, and U. H. Koszinowski.** 1989. Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4+ T lymphocytes. *J Exp Med* **169**:1199-212.
266. **Ju, S. T., D. J. Panka, H. Cui, R. Ettinger, M. el-Khatib, D. H. Sherr, B. Z. Stanger, and A. Marshak-Rothstein.** 1995. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature* **373**:444-8.
267. **Kagi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein.** 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* **265**:528-30.

268. **Kalams, S. A., S. P. Buchbinder, E. S. Rosenberg, J. M. Billingsley, D. S. Colbert, N. G. Jones, A. K. Shea, A. K. Trocha, and B. D. Walker.** 1999. Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection. *J Virol* **73**:6715-20.
269. **Kalinkovich, A., Z. Weisman, Z. Greenberg, J. Nahmias, S. Eitan, M. Stein, and Z. Bentwich.** 1998. Decreased CD4 and increased CD8 counts with T cell activation is associated with chronic helminth infection. *Clin Exp Immunol* **114**:414-21.
270. **Kanki, P. J., F. Barin, S. M'Boup, J. S. Allan, J. L. Romet-Lemonne, R. Marlink, M. F. McLane, T. H. Lee, B. Arbeille, F. Denis, and et al.** 1986. New human T-lymphotropic retrovirus related to simian T-lymphotropic virus type III (STLV-IIIAGM). *Science* **232**:238-43.
271. **Kanki, P. J., K. U. Travers, M. B. S, C. C. Hsieh, R. G. Marlink, N. A. Gueye, T. Siby, I. Thior, M. Hernandez-Avila, J. L. Sankale, and et al.** 1994. Slower heterosexual spread of HIV-2 than HIV-1. *Lancet* **343**:943-6.
272. **Kao, S. Y., A. F. Calman, P. A. Luciw, and B. M. Peterlin.** 1987. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* **330**:489-93.
273. **Kassu, A., A. Tsegaye, B. Petros, D. Wolday, E. Hailu, T. Tilahun, B. Hailu, M. T. Roos, A. L. Fontanet, D. Hamann, and T. F. De Wit.** 2001. Distribution of lymphocyte subsets in healthy human immunodeficiency virus-negative adult Ethiopians from two geographic locales. *Clin Diagn Lab Immunol* **8**:1171-6.
274. **Kassu, A., A. Tsegaye, D. Wolday, B. Petros, M. Aklilu, E. J. Sanders, A. L. Fontanet, D. Van Baarle, D. Hamann, and T. F. De Wit.** 2003. Role of incidental and/or cured intestinal parasitic infections on profile of CD4+ and CD8+ T cell subsets and activation status in HIV-1 infected and uninfected adult Ethiopians. *Clin Exp Immunol* **132**:113-9.
275. **Kaul, R., T. Dong, F. A. Plummer, J. Kimani, T. Rostron, P. Kiama, E. Njagi, E. Irungu, B. Farah, J. Oyugi, R. Chakraborty, K. S. MacDonald, J. J. Bwayo, A. McMichael, and S. L. Rowland-Jones.** 2001. CD8(+) lymphocytes respond to different HIV epitopes in seronegative and infected subjects. *J Clin Invest* **107**:1303-10.
276. **Kaul, R., F. A. Plummer, J. Kimani, T. Dong, P. Kiama, T. Rostron, E. Njagi, K. S. MacDonald, J. J. Bwayo, A. J. McMichael, and S. L. Rowland-Jones.** 2000. HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. *J Immunol* **164**:1602-11.
277. **Kaul, R., S. L. Rowland-Jones, J. Kimani, K. Fowke, T. Dong, P. Kiama, J. Rutherford, E. Njagi, F. Mwangi, T. Rostron, J. Onyango, J. Oyugi, K. S. MacDonald, J. J. Bwayo, and F. A. Plummer.** 2001. New insights into HIV-1 specific cytotoxic T-lymphocyte responses in exposed, persistently seronegative Kenyan sex workers. *Immunol Lett* **79**:3-13.
278. **Keever, C. A., M. Abu-Hajir, W. Graf, P. McFadden, P. Prichard, J. O'Brien, and N. Flomenberg.** 1995. Characterization of the alloreactivity and anti-leukemia reactivity of cord blood mononuclear cells. *Bone Marrow Transplant* **15**:407-19.
279. **Kestens, L., G. Vanham, C. Vereecken, M. Vandenbruaene, G. Vercauteren, R. L. Colebunders, and P. L. Gigase.** 1994. Selective increase

- of activation antigens HLA-DR and CD38 on CD4+ CD45RO+ T lymphocytes during HIV-1 infection. *Clin Exp Immunol* **95**:436-41.
280. **Kim, J. M., and A. Rudensky.** 2006. The role of the transcription factor Foxp3 in the development of regulatory T cells. *Immunol Rev* **212**:86-98.
 281. **Kim, S. Y., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. *J Virol* **63**:3708-13.
 282. **Kim, Y. H., S. H. Chang, J. H. Kwon, and S. S. Rhee.** 1999. HIV-1 Nef plays an essential role in two independent processes in CD4 down-regulation: dissociation of the CD4-p56(lck) complex and targeting of CD4 to lysosomes. *Virology* **257**:208-19.
 283. **Kitani, A., K. Chua, K. Nakamura, and W. Strober.** 2000. Activated self-MHC-reactive T cells have the cytokine phenotype of Th3/T regulatory cell 1 T cells. *J Immunol* **165**:691-702.
 284. **Kitchen, B. J., H. D. Engler, V. J. Gill, D. Marshall, S. M. Steinberg, P. A. Pizzo, and B. U. Mueller.** 1997. Cytomegalovirus infection in children with human immunodeficiency virus infection. *Pediatr Infect Dis J* **16**:358-63.
 285. **Kliks, S. C., D. W. Wara, D. V. Landers, and J. A. Levy.** 1994. Features of HIV-1 that could influence maternal-child transmission. *Jama* **272**:467-74.
 286. **Klimkait, T., K. Strebel, M. D. Hoggan, M. A. Martin, and J. M. Orenstein.** 1990. The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. *J Virol* **64**:621-9.
 287. **Knipe, D. M., P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, R. Roizman, and S. E. Straus.** 2007. *Fields Virology*, 5th ed. Lippincott Williams & Wilkins.
 288. **Komori, T., A. Okada, V. Stewart, and F. W. Alt.** 1993. Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes. *Science* **261**:1171-5.
 289. **Korber, B., M. Muldoon, J. Theiler, F. Gao, R. Gupta, A. Lapedes, B. H. Hahn, S. Wolinsky, and T. Bhattacharya.** 2000. Timing the ancestor of the HIV-1 pandemic strains. *Science* **288**:1789-96.
 290. **Kostense, S., G. S. Ogg, E. H. Manting, G. Gillespie, J. Joling, K. Vandenberghe, E. Z. Veenhof, D. van Baarle, S. Jurriaans, M. R. Klein, and F. Miedema.** 2001. High viral burden in the presence of major HIV-specific CD8(+) T cell expansions: evidence for impaired CTL effector function. *Eur J Immunol* **31**:677-86.
 291. **Kostense, S., K. Vandenberghe, J. Joling, D. Van Baarle, N. Nanlohy, E. Manting, and F. Miedema.** 2002. Persistent numbers of tetramer+ CD8(+) T cells, but loss of interferon-gamma+ HIV-specific T cells during progression to AIDS. *Blood* **99**:2505-11.
 292. **Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* **68**:4650-5.
 293. **Kovacs, A., M. Schluchter, K. Easley, G. Demmler, W. Shearer, P. La Russa, J. Pitt, E. Cooper, J. Goldfarb, D. Hodes, M. Kattan, and K. McIntosh.** 1999. Cytomegalovirus infection and HIV-1 disease progression in infants born to HIV-1-infected women. *Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted HIV Infection Study Group. N Engl J Med* **341**:77-84.

294. **Kovacsovics-Bankowski, M., and K. L. Rock.** 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* **267**:243-6.
295. **Kreiss, J., D. M. Willerford, M. Hensel, W. Emonyi, F. Plummer, J. Ndinya-Achola, P. L. Roberts, J. Hoskyn, S. Hillier, and N. Kiviat.** 1994. Association between cervical inflammation and cervical shedding of human immunodeficiency virus DNA. *J Infect Dis* **170**:1597-601.
296. **Kreiss, J. K., D. Koech, F. A. Plummer, K. K. Holmes, M. Lightfoote, P. Piot, A. R. Ronald, J. O. Ndinya-Achola, L. J. D'Costa, P. Roberts, and et al.** 1986. AIDS virus infection in Nairobi prostitutes. Spread of the epidemic to East Africa. *N Engl J Med* **314**:414-8.
297. **Krieger, J. N., R. W. Coombs, A. C. Collier, S. O. Ross, K. Chaloupka, D. K. Cummings, V. L. Murphy, and L. Corey.** 1991. Recovery of human immunodeficiency virus type 1 from semen: minimal impact of stage of infection and current antiviral chemotherapy. *J Infect Dis* **163**:386-8.
298. **Kuhn, L., A. Coutsoudis, D. Moodley, N. Mngqundaniso, D. Trabattoni, G. M. Shearer, M. Clerici, and H. M. Coovadia.** 2001. Interferon-gamma and interleukin-10 production among HIV-1-infected and uninfected infants of HIV-1-infected mothers. *Pediatr Res* **50**:412-6.
299. **Kuhn, L., A. Coutsoudis, D. Moodley, D. Trabattoni, N. Mngqundaniso, G. M. Shearer, M. Clerici, H. M. Coovadia, and Z. Stein.** 2001. T-helper cell responses to HIV envelope peptides in cord blood: protection against intrapartum and breast-feeding transmission. *Aids* **15**:1-9.
300. **Kuhn, L., S. Meddows-Taylor, G. Gray, and C. Tiemessen.** 2002. Human immunodeficiency virus (HIV)-specific cellular immune responses in newborns exposed to HIV in utero. *Clin Infect Dis* **34**:267-76.
301. **Kuhn, L., T. M. Meyers, S. Meddows-Taylor, K. Simmank, G. G. Sherman, and C. T. Tiemessen.** 2001. Human immunodeficiency virus type 1 envelope-stimulated interleukin-2 production and survival of infected children with severe and mild clinical disease. *J Infect Dis* **184**:691-8.
302. **Kuroda, M. J., J. E. Schmitz, W. A. Charini, C. E. Nickerson, M. A. Lifton, C. I. Lord, M. A. Forman, and N. L. Letvin.** 1999. Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. *J Immunol* **162**:5127-33.
303. **Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**:648-59.
304. **Lagaye, S., M. Derrien, E. Menu, C. Coito, E. Tresoldi, P. Mauciere, G. Scarlatti, G. Chaouat, F. Barre-Sinoussi, and M. Bomsel.** 2001. Cell-to-cell contact results in a selective translocation of maternal human immunodeficiency virus type 1 quasiespecies across a trophoblastic barrier by both transcytosis and infection. *J Virol* **75**:4780-91.
305. **Lalvani, A., R. Brookes, S. Hambleton, W. J. Britton, A. V. S. Hill, and A. J. McMichael.** 1997. Rapid Effector Function in CD8+ Memory T Cells. *J Exp Med* **186**:859-865.
306. **Lanari, M., T. Lazzarotto, V. Venturi, I. Papa, L. Gabrielli, B. Guerra, M. P. Landini, and G. Faldella.** 2006. Neonatal cytomegalovirus blood load

and risk of sequelae in symptomatic and asymptomatic congenitally infected newborns. *Pediatrics* **117**:e76-83.

307. **Landau, N. R., M. Warton, and D. R. Littman.** 1988. The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4. *Nature* **334**:159-62.
308. **Lathey, J. L., D. H. Spector, and S. A. Spector.** 1994. Human cytomegalovirus-mediated enhancement of human immunodeficiency virus type-1 production in monocyte-derived macrophages. *Virology* **199**:98-104.
309. **Latron, F., R. Moots, J. B. Rothbard, T. P. Garrett, J. L. Strominger, and A. McMichael.** 1991. Positioning of a peptide in the cleft of HLA-A2 by complementing amino acid changes. *Proc Natl Acad Sci U S A* **88**:11325-9.
310. **Lavreys, L., J. M. Baeten, V. Chohan, R. S. McClelland, W. M. Hassan, B. A. Richardson, K. Mandalia, J. O. Ndinya-Achola, and J. Overbaugh.** 2006. Higher set point plasma viral load and more-severe acute HIV type 1 (HIV-1) illness predict mortality among high-risk HIV-1-infected African women. *Clin Infect Dis* **42**:1333-9.
311. **Lazarotto, T., L. Gabrielli, M. Lanari, B. Guerra, T. Bellucci, M. Sassi, and M. P. Landini.** 2004. Congenital cytomegalovirus infection: recent advances in the diagnosis of maternal infection. *Hum Immunol* **65**:410-5.
312. **Le, N. T., and D. R. Richardson.** 2002. The role of iron in cell cycle progression and the proliferation of neoplastic cells. *Biochim Biophys Acta* **1603**:31-46.
313. **Le Priol, Y., D. Puthier, C. Lecureuil, C. Combadiere, P. Debre, C. Nguyen, and B. Combadiere.** 2006. High cytotoxic and specific migratory potencies of senescent CD8⁺ CD57⁺ cells in HIV-infected and uninfected individuals. *J Immunol* **177**:5145-54.
314. **Lee, G. R., P. E. Fields, and R. A. Flavell.** 2001. Regulation of IL-4 gene expression by distal regulatory elements and GATA-3 at the chromatin level. *Immunity* **14**:447-59.
315. **Lee, J. K., G. Stewart-Jones, T. Dong, K. Harlos, K. Di Gleria, L. Dorrell, D. C. Douek, P. A. van der Merwe, E. Y. Jones, and A. J. McMichael.** 2004. T cell cross-reactivity and conformational changes during TCR engagement. *J Exp Med* **200**:1455-66.
316. **Legrand, F. A., D. F. Nixon, C. P. Loo, E. Ono, J. M. Chapman, M. Miyamoto, R. S. Diaz, A. M. Santos, R. C. Succi, J. Abadi, M. G. Rosenberg, M. I. de Moraes-Pinto, and E. G. Kallas.** 2006. Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants and neonates revealed after regulatory T cell removal. *PLoS ONE* **1**:e102.
317. **Lehman, D. A., and C. Farquhar.** 2007. Biological mechanisms of vertical human immunodeficiency virus (HIV-1) transmission. *Rev Med Virol.*
318. **Lepage, P., R. Spira, S. Kalibala, K. Pillay, C. Giaquinto, K. Castetbon, C. Osborne, C. Courpotin, and F. Dabis.** 1998. Care of human immunodeficiency virus-infected children in developing countries. International Working Group on Mother-to-Child Transmission of HIV. *Pediatr Infect Dis J* **17**:581-6.
319. **Leslie, A. J., K. J. Pfaffertott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker, and**

- P. J. Goulder. 2004. HIV evolution: CTL escape mutation and reversion after transmission. *Nat Med* 10:282-9.
320. Letvin, N. L., J. R. Mascola, Y. Sun, D. A. Gorgone, A. P. Buzby, L. Xu, Z. Y. Yang, B. Chakrabarti, S. S. Rao, J. E. Schmitz, D. C. Montefiori, B. R. Barker, F. L. Bookstein, and G. J. Nabel. 2006. Preserved CD4⁺ central memory T cells and survival in vaccinated SIV-challenged monkeys. *Science* 312:1530-3.
321. Levacher, M., F. Hulstaert, S. Tallet, S. Ullery, J. J. Pocidalo, and B. A. Bach. 1992. The significance of activation markers on CD8 lymphocytes in human immunodeficiency syndrome: staging and prognostic value. *Clin Exp Immunol* 90:376-82.
322. Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* 225:840-2.
323. Levy, J. A., F. Hsueh, D. J. Blackbourn, D. Wara, and P. S. Weintrub. 1998. CD8 cell noncytotoxic antiviral activity in human immunodeficiency virus-infected and -uninfected children. *J Infect Dis* 177:470-2.
324. Lewis, S. H., C. Reynolds-Kohler, H. E. Fox, and J. A. Nelson. 1990. HIV-1 in trophoblastic and villous Hofbauer cells, and haematological precursors in eight-week fetuses. *Lancet* 335:565-8.
325. Li, Q., L. Duan, J. D. Estes, Z. M. Ma, T. Rourke, Y. Wang, C. Reilly, J. Carlis, C. J. Miller, and A. T. Haase. 2005. Peak SIV replication in resting memory CD4⁺ T cells depletes gut lamina propria CD4⁺ T cells. *Nature* 434:1148-52.
326. Lichterfeld, M., D. E. Kaufmann, X. G. Yu, S. K. Mui, M. M. Addo, M. N. Johnston, D. Cohen, G. K. Robbins, E. Pae, G. Alter, A. Wurcel, D. Stone, E. S. Rosenberg, B. D. Walker, and M. Altfeld. 2004. Loss of HIV-1-specific CD8⁺ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4⁺ T cells. *J Exp Med* 200:701-12.
327. Lieberman, J., L. A. Trimble, R. S. Friedman, J. Lisziewicz, F. Lori, P. Shankar, and H. Jessen. 1999. Expansion of CD57 and CD62L-CD45RA⁺ CD8 T lymphocytes correlates with reduced viral plasma RNA after primary HIV infection. *Aids* 13:891-9.
328. Liebes, L., S. Mendoza, D. Wilson, and J. Dancis. 1990. Transfer of zidovudine (AZT) by human placenta. *J Infect Dis* 161:203-7.
329. Lifson, A. R., and M. F. Rogers. 1986. Vertical transmission of human immunodeficiency virus. *Lancet* 2:337.
330. Liu, H., D. Chao, E. E. Nakayama, H. Taguchi, M. Goto, X. Xin, J. K. Takamatsu, H. Saito, Y. Ishikawa, T. Akaza, T. Juji, Y. Takebe, T. Ohishi, K. Fukutake, Y. Maruyama, S. Yashiki, S. Sonoda, T. Nakamura, Y. Nagai, A. Iwamoto, and T. Shioda. 1999. Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression. *Proc Natl Acad Sci U S A* 96:4581-5.
331. Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup, and N. R. Landau. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 86:367-77.
332. Liu, Z., W. G. Cumberland, L. E. Hultin, H. E. Prince, R. Detels, and J. V. Giorgi. 1997. Elevated CD38 antigen expression on CD8⁺ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and

- death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. *J Acquir Immune Defic Syndr Hum Retrovirol* **16**:83-92.
333. **Lohman, B. L., J. A. Slyker, B. A. Richardson, C. Farquhar, J. M. Mabuka, C. Crudder, T. Dong, E. Obimbo, D. Mbori-Ngacha, J. Overbaugh, S. Rowland-Jones, and G. John-Stewart.** 2005. Longitudinal assessment of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon responses during the first year of life in HIV-1-infected infants. *J Virol* **79**:8121-30.
 334. **Looney, R. J., A. Falsey, D. Campbell, A. Torres, J. Kolassa, C. Brower, R. McCann, M. Menegus, K. McCormick, M. Frampton, W. Hall, and G. N. Abraham.** 1999. Role of cytomegalovirus in the T cell changes seen in elderly individuals. *Clin Immunol* **90**:213-9.
 335. **Lopez, F., F. Belloc, F. Lacombe, P. Dumain, J. Reiffers, P. Bernard, and M. R. Boisseau.** 1991. Modalities of synthesis of Ki67 antigen during the stimulation of lymphocytes. *Cytometry* **12**:42-9.
 336. **Lukashov, V. V., and J. Goudsmit.** 1998. HIV heterogeneity and disease progression in AIDS: a model of continuous virus adaptation. *Aids* **12 Suppl A**:S43-52.
 337. **Luzuriaga, K., D. Holmes, A. Hereema, J. Wong, D. L. Panicali, and J. L. Sullivan.** 1995. HIV-1-specific cytotoxic T lymphocyte responses in the first year of life. *J Immunol* **154**:433-43.
 338. **Luzuriaga, K., R. A. Koup, C. A. Pikora, D. B. Brettler, and J. L. Sullivan.** 1991. Deficient human immunodeficiency virus type 1-specific cytotoxic T cell responses in vertically infected children. *J Pediatr* **119**:230-6.
 339. **Luzuriaga, K., P. McQuilken, A. Alimenti, M. Somasundaran, R. A. Hesselton, and J. L. Sullivan.** 1993. Early viremia and immune responses in vertical HIV type 1 infection. *J Infectious Diseases* **167**:1008-1013.
 340. **MacDonald, K. S., J. Embree, S. Njenga, N. J. Nagelkerke, I. Ngatia, Z. Mohammed, B. H. Barber, J. Ndinya-Achola, J. Bwayo, and F. A. Plummer.** 1998. Mother-child class I HLA concordance increases perinatal human immunodeficiency virus type 1 transmission. *J Infect Dis* **177**:551-6.
 341. **Malavasi, F., A. Funaro, S. Roggero, A. Horenstein, L. Calosso, and K. Mehta.** 1994. Human CD38: a glycoprotein in search of a function. *Immunol Today* **15**:95-7.
 342. **Malim, M. H., J. Hauber, S. Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature* **338**:254-7.
 343. **Mandelbrot, L., J. Le Chenadec, A. Berrebi, A. Bongain, J. L. Benifla, J. F. Delfraissy, S. Blanche, and M. J. Mayaux.** 1998. Perinatal HIV-1 transmission: interaction between zidovudine prophylaxis and mode of delivery in the French Perinatal Cohort. *Jama* **280**:55-60.
 344. **Manfredi, R., and S. Sabbatani.** 2006. A novel antiretroviral class (fusion inhibitors) in the management of HIV infection. Present features and future perspectives of enfuvirtide (T-20). *Curr Med Chem* **13**:2369-84.
 345. **Marandin, A., A. Katz, E. Oksenhendler, M. Tulliez, F. Picard, W. Vainchenker, and F. Louache.** 1996. Loss of primitive hematopoietic progenitors in patients with human immunodeficiency virus infection. *Blood* **88**:4568-78.

346. **Marchant, A., V. Appay, M. Van Der Sande, N. Dulphy, C. Liesnard, M. Kidd, S. Kaye, O. Ojuola, G. M. Gillespie, A. L. Vargas Cuero, V. Cerundolo, M. Callan, K. P. McAdam, S. L. Rowland-Jones, C. Donner, A. J. McMichael, and H. Whittle.** 2003. Mature CD8(+) T lymphocyte response to viral infection during fetal life. *J Clin Invest* **111**:1747-55.
347. **Marechal, V., M. C. Prevost, C. Petit, E. Perret, J. M. Heard, and O. Schwartz.** 2001. Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis. *J Virol* **75**:11166-77.
348. **Markosyan, R. M., F. S. Cohen, and G. B. Melikyan.** 2003. HIV-1 envelope proteins complete their folding into six-helix bundles immediately after fusion pore formation. *Mol Biol Cell* **14**:926-38.
349. **Marlink, R., P. Kanki, I. Thior, K. Travers, G. Eisen, T. Siby, I. Traore, C. C. Hsieh, M. C. Dia, E. H. Gueye, and et al.** 1994. Reduced rate of disease development after HIV-2 infection as compared to HIV-1. *Science* **265**:1587-90.
350. **Martin, A. W., K. Brady, S. I. Smith, D. DeCoste, D. V. Page, A. Malpica, B. Wolf, and R. S. Neiman.** 1992. Immunohistochemical localization of human immunodeficiency virus p24 antigen in placental tissue. *Hum Pathol* **23**:411-4.
351. **Martinez-Steele, E., A. A. Awasana, T. Corrah, S. Sabally, M. van der Sande, A. Jaye, T. Togun, R. Sarge-Njie, S. J. McConkey, H. Whittle, and M. F. Schim van der Loeff.** 2007. Is HIV-2- induced AIDS different from HIV-1-associated AIDS? Data from a West African clinic. *Aids* **21**:317-24.
352. **Masur, H., M. A. Michelis, J. B. Greene, I. Onorato, R. A. Stouwe, R. S. Holzman, G. Wormser, L. Brettman, M. Lange, H. W. Murray, and S. Cunningham-Rundles.** 1981. An outbreak of community-acquired *Pneumocystis carinii* pneumonia: initial manifestation of cellular immune dysfunction. *N Engl J Med* **305**:1431-8.
353. **Masur, H., F. P. Ognibene, R. Yarchoan, J. H. Shelhamer, B. F. Baird, W. Travis, A. F. Suffredini, L. Deyton, J. A. Kovacs, J. Falloon, and et al.** 1989. CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection. *Ann Intern Med* **111**:223-31.
354. **Mattern, C. F., K. Murray, A. Jensen, H. Farzadegan, J. Pang, and J. F. Modlin.** 1992. Localization of human immunodeficiency virus core antigen in term human placentas. *Pediatrics* **89**:207-9.
355. **Mbori-Ngacha, D., R. Nduati, G. John, M. Reilly, B. Richardson, A. Mwatha, J. Ndinya-Achola, J. Bwayo, and J. Kreiss.** 2001. Morbidity and mortality in breastfed and formula-fed infants of HIV-1- infected women: A randomized clinical trial. *Jama* **286**:2413-20.
356. **McBlane, J. F., D. C. van Gent, D. A. Ramsden, C. Romeo, C. A. Cuomo, M. Gellert, and M. A. Oettinger.** 1995. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell* **83**:387-95.
357. **McCloskey, T. W., V. Haridas, R. Pahwa, and S. Pahwa.** 2001. Human immunodeficiency virus gag and pol-specific CD8 T cells in perinatal HIV infection. *Cytometry* **46**:265-70.
358. **McCloskey, T. W., N. Oyaizu, S. Bakshi, R. Kowalski, N. Kohn, and S. Pahwa.** 1998. CD95 expression and apoptosis during pediatric HIV infection: early upregulation of CD95 expression. *Clin Immunol Immunopathol* **87**:33-41.

359. **McFarland, E. J., P. A. Harding, D. Luckey, and e. al.** 1994. High frequency of gag and envelope specific CTL precursors in children with vertically acquired HIV-1 infection. *J Infect Dis* **170**.
360. **McMichael, A. J., A. Ting, H. J. Zweerink, and B. A. Askonas.** 1977. HLA restriction of cell-mediated lysis of influenza virus-infected human cells. *Nature* **270**:524-6.
361. **Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr.** 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**:394-7.
362. **Mehandru, S., M. A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, and M. Markowitz.** 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* **200**:761-70.
363. **Mehta, K., U. Shahid, and F. Malavasi.** 1996. Human CD38, a cell-surface protein with multiple functions. *Faseb J* **10**:1408-17.
364. **Melikyan, G. B., R. M. Markosyan, H. Hemmati, M. K. Delmedico, D. M. Lambert, and F. S. Cohen.** 2000. Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *J Cell Biol* **151**:413-23.
365. **Mellors, J. W., A. Munoz, J. V. Giorgi, J. B. Margolick, C. J. Tassoni, P. Gupta, L. A. Kingsley, J. A. Todd, A. J. Saah, R. Detels, J. P. Phair, and C. R. Rinaldo, Jr.** 1997. Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. *Ann Intern Med* **126**:946-54.
366. **Mellors, J. W., C. R. Rinaldo, Jr., P. Gupta, R. M. White, J. A. Todd, and L. A. Kingsley.** 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* **272**:1167-70.
367. **Menu, E., F. X. Mbopi-Keou, S. Lagaye, S. Pissard, P. Mauciere, G. Scarlatti, J. Martin, M. Goossens, G. Chaouat, and F. Barre-Sinoussi.** 1999. Selection of maternal human immunodeficiency virus type 1 variants in human placenta. European Network for In Utero Transmission of HIV-1. *J Infect Dis* **179**:44-51.
368. **Mermin, J. H., M. Holodniy, D. A. Katzenstein, and T. C. Merigan.** 1991. Detection of human immunodeficiency virus DNA and RNA in semen by the polymerase chain reaction. *J Infect Dis* **164**:769-72.
369. **Messele, T., M. Abdulkadir, A. L. Fontanet, B. Petros, D. Hamann, M. Koot, M. T. Roos, P. T. Schellekens, F. Miedema, and T. F. Rinke de Wit.** 1999. Reduced naive and increased activated CD4 and CD8 cells in healthy adult Ethiopians compared with their Dutch counterparts. *Clin Exp Immunol* **115**:443-50.
370. **Meyers, T. M., L. Kuhn, S. Meddows-Taylor, K. Simmank, G. G. Sherman, and C. T. Tiemessen.** 2000. T-helper cell responses among HIV-infected children in Soweto, South Africa. *Ann N Y Acad Sci* **918**:373-6.
371. **Michaelsson, J., J. E. Mold, J. M. McCune, and D. F. Nixon.** 2006. Regulation of T cell responses in the developing human fetus. *J Immunol* **176**:5741-8.
372. **Miles, D. J., M. van der Sande, D. Jeffries, S. Kaye, J. Ismaili, O. Ojuola, M. Sanneh, E. S. Touray, P. Waight, S. Rowland-Jones, H. Whittle, and A. Marchant.** 2007. Cytomegalovirus infection in gambian infants leads to profound CD8 T-cell differentiation. *J Virol* **81**:5766-76.

373. **Mims, C. A., and R. V. Blanden.** 1972. Antiviral action of immune lymphocytes in mice infected with lymphocytic choriomeningitis virus. *Infect Immun* 6:695-8.
374. **Minkoff, H., D. N. Burns, S. Landesman, J. Youchah, J. J. Goedert, R. P. Nugent, L. R. Muenz, and A. D. Willoughby.** 1995. The relationship of the duration of ruptured membranes to vertical transmission of human immunodeficiency virus. *Am J Obstet Gynecol* 173:585-9.
375. **Mofenson, L. M., J. S. Lambert, E. R. Stiehlm, J. Bethel, W. A. Meyer, 3rd, J. Whitehouse, J. Moye, Jr., P. Reichelderfer, D. R. Harris, M. G. Fowler, B. J. Mathieson, and G. J. Nemo.** 1999. Risk factors for perinatal transmission of human immunodeficiency virus type 1 in women treated with zidovudine. Pediatric AIDS Clinical Trials Group Study 185 Team. *N Engl J Med* 341:385-93.
376. **Mognetti, B., M. Moussa, J. Croitoru, E. Menu, D. Dormont, P. Roques, and G. Chaouat.** 2000. HIV-1 co-receptor expression on trophoblastic cells from early placentas and permissivity to infection by several HIV-1 primary isolates. *Clin Exp Immunol* 119:486-92.
377. **Montes, M., D. E. Lewis, C. Sanchez, D. L. de Castilla, E. A. Graviss, C. Seas, E. Gotuzzo, and A. C. White, Jr.** 2006. Foxp3+ regulatory T cells in antiretroviral-naïve HIV patients. *Aids* 20:1669-71.
378. **Moore, R. D., and R. E. Chaisson.** 1996. Natural history of opportunistic disease in an HIV-infected urban clinical cohort. *Ann Intern Med* 124:633-42.
379. **Moss, A. R., P. Bacchetti, D. Osmond, W. Krampf, R. E. Chaisson, D. Stites, J. Wilber, J. P. Allain, and J. Carlson.** 1988. Seropositivity for HIV and the development of AIDS or AIDS related condition: three year follow up of the San Francisco General Hospital cohort. *Br Med J (Clin Res Ed)* 296:745-50.
380. **Mostad, S. B., J. K. Kreiss, A. J. Ryncarz, J. Overbaugh, K. Mandalia, B. Chohan, J. Ndinya-Achola, J. J. Bwayo, and L. Corey.** 1999. Cervical shedding of cytomegalovirus in human immunodeficiency virus type 1-infected women. *J Med Virol* 59:469-73.
381. **Motley, D., M. P. Meyer, R. A. King, and G. J. Naus.** 1996. Determination of lymphocyte immunophenotypic values for normal full-term cord blood. *Am J Clin Pathol* 105:38-43.
382. **Mphatswe, W., N. Blanckenberg, G. Tudor-Williams, A. Prendergast, C. Thobakgale, N. Mkhwanazi, N. McCarthy, B. D. Walker, P. Kiepiela, and P. Goulder.** 2007. High frequency of rapid immunological progression in African infants infected in the era of perinatal HIV prophylaxis. *Aids* 21:1253-61.
383. **Mullen, A. C., F. A. High, A. S. Hutchins, H. W. Lee, A. V. Villarino, D. M. Livingston, A. L. Kung, N. Cereb, T. P. Yao, S. Y. Yang, and S. L. Reiner.** 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292:1907-10.
384. **Mundy, D. C., R. F. Schinazi, A. R. Gerber, A. J. Nahmias, and H. W. Randall, Jr.** 1987. Human immunodeficiency virus isolated from amniotic fluid. *Lancet* 2:459-60.
385. **Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed.** 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8:177-87.

386. **Muro-Cacho, C. A., G. Pantaleo, and A. S. Fauci.** 1995. Analysis of apoptosis in lymph nodes of HIV-infected persons. Intensity of apoptosis correlates with the general state of activation of the lymphoid tissue and not with stage of disease or viral burden. *J Immunol* **154**:5555-66.
387. **Musey, L., J. Hughes, T. Schacker, T. Shea, L. Corey, and M. J. McElrath.** 1997. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *N Engl J Med* **337**:1267-74.
388. **Musey, L. K., J. N. Krieger, J. P. Hughes, T. W. Schacker, L. Corey, and M. J. McElrath.** 1999. Early and persistent human immunodeficiency virus type 1 (HIV-1)-specific T helper dysfunction in blood and lymph nodes following acute HIV-1 infection. *J Infect Dis* **180**:278-84.
389. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* **326**:711-3.
390. **Nduati, R., G. John, D. Mbori-Ngacha, B. Richardson, J. Overbaugh, A. Mwatha, J. Ndinya-Achola, J. Bwayo, F. E. Onyango, J. Hughes, and J. Kreiss.** 2000. Effect of breastfeeding and formula feeding on transmission of HIV-1: a randomized clinical trial. *Jama* **283**:1167-74.
391. **Nduati, R. W., G. C. John, B. A. Richardson, J. Overbaugh, M. Welch, J. Ndinya-Achola, S. Moses, K. Holmes, F. Onyango, and J. K. Kreiss.** 1995. Human immunodeficiency virus type 1-infected cells in breast milk: association with immunosuppression and vitamin A deficiency. *J Infect Dis* **172**:1461-8.
392. **Neckers, L. M., and J. Cossman.** 1983. Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. *Proc Natl Acad Sci U S A* **80**:3494-8.
393. **Neilson, J. R., G. C. John, J. K. Carr, P. Lewis, J. K. Kreiss, S. Jackson, R. W. Nduati, D. Mbori-Ngacha, D. D. Panteleeff, S. Bodrug, C. Giachetti, M. A. Bott, B. A. Richardson, J. Bwayo, J. Ndinya-Achola, and J. Overbaugh.** 1999. Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. *J Virol* **73**:4393-403.
394. **Neuberger, M. S., M. R. Ehrenstein, C. Rada, J. Sale, F. D. Batista, G. Williams, and C. Milstein.** 2000. Memory in the B-cell compartment: antibody affinity maturation. *Philos Trans R Soc Lond B Biol Sci* **355**:357-60.
395. **Newell, M. L., H. Coovadia, M. Cortina-Borja, N. Rollins, P. Gaillard, and F. Dabis.** 2004. Mortality of infected and uninfected infants born to HIV-infected mothers in Africa: a pooled analysis. *Lancet* **364**:1236-43.
396. **Nigro, G., A. Krzysztofciak, G. C. Gattinara, T. Mango, M. Mazzocco, M. A. Porcaro, S. Provvedi, and J. C. Booth.** 1996. Rapid progression of HIV disease in children with cytomegalovirus DNAemia. *Aids* **10**:1127-33.
397. **Nuvor, S. V., M. van der Sande, S. Rowland-Jones, H. Whittle, and A. Jaye.** 2006. Natural killer cell function is well preserved in asymptomatic human immunodeficiency virus type 2 (HIV-2) infection but similar to that of HIV-1 infection when CD4 T-cell counts fall. *J Virol* **80**:2529-38.
398. **Nyandiko, W. M., S. Ayaya, E. Nabakwe, C. Tenge, J. E. Sidle, C. T. Yiannoutsos, B. Musick, K. Wools-Kaloustian, and W. M. Tierney.** 2006. Outcomes of HIV-infected orphaned and non-orphaned children on

- antiretroviral therapy in western Kenya. *J Acquir Immune Defic Syndr* 43:418-25.
399. **O'Brien, T. R., W. A. Blattner, D. Waters, E. Eyster, M. W. Hilgartner, A. R. Cohen, N. Luban, A. Hatzakis, L. M. Aledort, P. S. Rosenberg, W. J. Miley, B. L. Kroner, and J. J. Goedert.** 1996. Serum HIV-1 RNA levels and time to development of AIDS in the Multicenter Hemophilia Cohort Study. *Jama* 276:105-10.
 400. **O'Garra, A., and N. Arai.** 2000. The molecular basis of T helper 1 and T helper 2 cell differentiation. *Trends Cell Biol* 10:542-50.
 401. **Obimbo, E. M., D. A. Mbori-Ngacha, J. O. Ochieng, B. A. Richardson, P. A. Otieno, R. Bosire, C. Farquhar, J. Overbaugh, and G. C. John-Stewart.** 2004. Predictors of early mortality in a cohort of human immunodeficiency virus type 1-infected african children. *Pediatr Infect Dis J* 23:536-43.
 402. **Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D. D. Ho, D. F. Nixon, and A. J. McMichael.** 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279:2103-6.
 403. **Ogg, G. S., and A. J. McMichael.** 1999. Quantitation of antigen-specific CD8+ T-cell responses. *Immunol Lett* 66:77-80.
 404. **Ohoka, Y., T. Kuwata, Y. Tozawa, Y. Zhao, M. Mukai, Y. Motegi, R. Suzuki, M. Yokoyama, and M. Iwata.** 1996. In vitro differentiation and commitment of CD4+ CD8+ thymocytes to the CD4 lineage, without TCR engagement. *Int Immunol* 8:297-306.
 405. **Oliva, A., A. L. Kinter, M. Vaccarezza, A. Rubbert, A. Catanzaro, S. Moir, J. Monaco, L. Ehler, S. Mizell, R. Jackson, Y. Li, J. W. Romano, and A. S. Fauci.** 1998. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. *J Clin Invest* 102:223-31.
 406. **Olsson, J., A. Wikby, B. Johansson, S. Lofgren, B. O. Nilsson, and F. G. Ferguson.** 2000. Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. *Mech Ageing Dev* 121:187-201.
 407. **Otieno, P. A., E. R. Brown, D. A. Mbori-Ngacha, R. W. Nduati, C. Farquhar, E. M. Obimbo, R. K. Bosire, S. Emery, J. Overbaugh, B. A. Richardson, and G. C. John-Stewart.** 2007. HIV-1 disease progression in breast-feeding and formula-feeding mothers: a prospective 2-year comparison of T cell subsets, HIV-1 RNA levels, and mortality. *J Infect Dis* 195:220-9.
 408. **Palella, F. J., Jr., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, and S. D. Holmberg.** 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* 338:853-60.
 409. **Palmer, B. E., N. Blyveis, A. P. Fontenot, and C. C. Wilson.** 2005. Functional and phenotypic characterization of CD57+CD4+ T cells and their association with HIV-1-induced T cell dysfunction. *J Immunol* 175:8415-23.
 410. **Palumbo, P. E., C. Raskino, S. Fiscus, S. Pahwa, M. G. Fowler, S. A. Spector, J. A. Englund, and C. J. Baker.** 1998. Predictive value of

- quantitative plasma HIV RNA and CD4+ lymphocyte count in HIV-infected infants and children. *Jama* **279**:756-61.
411. **Pantaleo, G., C. Graziosi, and A. S. Fauci.** 1993. New concepts in the immunopathogenesis of human immunodeficiency virus infection. *N Engl J Med* **328**:327-335.
 412. **Papagno, L., C. A. Spina, A. Marchant, M. Salio, N. Rufer, S. Little, T. Dong, G. Chesney, A. Waters, P. Easterbrook, P. R. Dunbar, D. Shepherd, V. Cerundolo, V. Emery, P. Griffiths, C. Conlon, A. J. McMichael, D. D. Richman, S. L. Rowland-Jones, and V. Appay.** 2004. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. *PLoS Biol* **2**:E20.
 413. **Parkin, N. T., M. Chamorro, and H. E. Varmus.** 1992. Human immunodeficiency virus type 1 gag-pol frameshifting is dependent on downstream mRNA secondary structure: demonstration by expression in vivo. *J Virol* **66**:5147-51.
 414. **Pass, R. F., S. Stagno, G. J. Myers, and C. A. Alford.** 1980. Outcome of symptomatic congenital cytomegalovirus infection: results of long-term longitudinal follow-up. *Pediatrics* **66**:758-62.
 415. **Patki, A. H., S. P. Zielske, S. F. Sieg, and M. M. Lederman.** 2000. Preferential S phase entry and apoptosis of CD4(+) T lymphocytes of HIV-1-infected patients after in vitro cultivation. *Clin Immunol* **97**:241-7.
 416. **Paulsson, K. M., M. Jevon, J. W. Wang, S. Li, and P. Wang.** 2006. The double lysine motif of tapasin is a retrieval signal for retention of unstable MHC class I molecules in the endoplasmic reticulum. *J Immunol* **176**:7482-8.
 417. **Pawelec, G., A. Akbar, C. Caruso, R. Effros, B. Grubeck-Loebenstien, and A. Wikby.** 2004. Is immunosenescence infectious? *Trends Immunol* **25**:406-10.
 418. **Pediatrics, A. A. o.** 1997. Red Book: Section 1. Active and Passive Immunization: Immunization in Special Clinical Circumstances. HighWire Press.
 419. **Peliska, J. A., and S. J. Benkovic.** 1992. Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. *Science* **258**:1112-8.
 420. **Petrovas, C., Y. M. Mueller, I. D. Dimitriou, P. M. Bojczuk, K. C. Mounzer, J. Witek, J. D. Altman, and P. D. Katsikis.** 2004. HIV-specific CD8+ T cells exhibit markedly reduced levels of Bcl-2 and Bcl-xL. *J Immunol* **172**:4444-53.
 421. **Phillips, R. E., S. Rowland-Jones, D. F. Nixon, F. M. Gotch, J. P. Edwards, A. O. Ogunlesi, J. G. Elvin, J. A. Rothbard, C. R. Bangham, C. R. Rizza, and et al.** 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* **354**:453-9.
 422. **Picker, L. J.** 2006. Immunopathogenesis of acute AIDS virus infection. *Curr Opin Immunol* **18**:399-405.
 423. **Pikora, C. A., J. L. Sullivan, D. Panicali, and K. Luzuriaga.** 1997. Early HIV-1 envelope-specific cytotoxic T lymphocyte responses in vertically infected infants. *J Exp Med* **185**:1153-61.
 424. **Pillay, K., A. Coutoudis, A. K. Agadzi-Naqvi, L. Kuhn, H. M. Coovadia, and E. N. Janoff.** 2001. Secretory leukocyte protease inhibitor in vaginal fluids and perinatal human immunodeficiency virus type 1 transmission. *J Infect Dis* **183**:653-6.

425. **Pillay, T., H. T. Zhang, J. W. Drijfhout, N. Robinson, H. Brown, M. Khan, J. Moodley, M. Adhikari, K. Pfafferott, M. E. Feeney, A. St John, E. C. Holmes, H. M. Coovadia, P. Klenerman, P. J. Goulder, and R. E. Phillips.** 2005. Unique acquisition of cytotoxic T-lymphocyte escape mutants in infant human immunodeficiency virus type 1 infection. *J Virol* **79**:12100-5.
426. **Pitcher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino, and L. J. Picker.** 1999. HIV-1-specific CD4⁺ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat Med* **5**:518-25.
427. **Pitisuttithum, P., P. Gilbert, M. Gurwith, W. Heyward, M. Martin, F. van Griensven, D. Hu, J. W. Tappero, and K. Choopanya.** 2006. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis* **194**:1661-71.
428. **Plaeger, S., H. Z. Bass, P. Nishanian, J. Thomas, N. Aziz, R. Detels, J. King, W. Cumberland, M. Kemeny, and J. L. Fahey.** 1999. The prognostic significance in HIV infection of immune activation represented by cell surface antigen and plasma activation marker changes. *Clin Immunol* **90**:238-46.
429. **Plaeger-Marshall, S., P. Hultin, J. Bertolli, S. O'Rourke, R. Kobayashi, A. L. Kobayashi, J. V. Giorgi, Y. Bryson, and E. R. Stiehm.** 1993. Activation and differentiation antigens on T cells of healthy, at-risk, and HIV-infected children. *J Acquir Immune Defic Syndr* **6**:984-93.
430. **Plaeger-Marshall, S., V. Isacescu, S. O'Rourke, J. Bertolli, Y. J. Bryson, and E. R. Stiehm.** 1994. T cell activation in pediatric AIDS pathogenesis: three-color immunophenotyping. *Clin Immunol Immunopathol* **71**:19-26.
431. **Polk, B. F., R. Fox, R. Brookmeyer, S. Kanchanaraksa, R. Kaslow, B. Visscher, C. Rinaldo, and J. Phair.** 1987. Predictors of the acquired immunodeficiency syndrome developing in a cohort of seropositive homosexual men. *N Engl J Med* **316**:61-6.
432. **Pollack, S., B. Fuad, and A. Etzioni.** 1993. CD4 T-lymphocytopenia without opportunistic infections in HIV-seronegative Ethiopian immigrants to Israel. *Lancet* **342**:50.
433. **Polycarpou, A., C. Ntais, B. T. Korber, H. A. Elrich, R. Winchester, P. Krogstad, S. Wolinsky, T. Rostron, S. L. Rowland-Jones, A. J. Ammann, and J. P. Ioannidis.** 2002. Association between maternal and infant class I and II HLA alleles and of their concordance with the risk of perinatal HIV type 1 transmission. *AIDS Res Hum Retroviruses* **18**:741-6.
434. **Prescott, S. L., C. Macaubas, B. J. Holt, T. B. Smallacombe, R. Loh, P. D. Sly, and P. G. Holt.** 1998. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* **160**:4730-7.
435. **Price, D. A., P. J. Goulder, P. Klenerman, A. K. Sewell, P. J. Easterbrook, M. Troop, C. R. Bangham, and R. E. Phillips.** 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc Natl Acad Sci U S A* **94**:1890-5.
436. **Prince, H. E., and E. R. Jensen.** 1991. Three-color cytofluorometric analysis of CD8 cell subsets in HIV-1 infection. *J Acquir Immune Defic Syndr* **4**:1227-32.

437. **Pryciak, P. M., and H. E. Varmus.** 1992. Nucleosomes, DNA-binding proteins, and DNA sequence modulate retroviral integration target site selection. *Cell* **69**:769-80.
438. **Reid, K. B., and R. R. Porter.** 1981. The proteolytic activation systems of complement. *Annu Rev Biochem* **50**:433-64.
439. **Reinherz, E. L., P. C. Kung, G. Goldstein, R. H. Levey, and S. F. Schlossman.** 1980. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. *Proc Natl Acad Sci U S A* **77**:1588-92.
440. **Reitter, J. N., R. E. Means, and R. C. Desrosiers.** 1998. A role for carbohydrates in immune evasion in AIDS. *Nat Med* **4**:679-84.
441. **Revello, M. G., M. Zavattoni, A. Sarasini, E. Percivalle, L. Simoncini, and G. Gerna.** 1998. Human cytomegalovirus in blood of immunocompetent persons during primary infection: prognostic implications for pregnancy. *J Infect Dis* **177**:1170-5.
442. **Rich, K. C., D. Brambilla, J. Pitt, J. Moye, E. Cooper, G. Hillyer, H. Mendez, M. G. Fowler, and A. Landay.** 1997. Lymphocyte phenotyping in infants: maturation of lymphocyte subpopulations and the effects of HIV infection. *Clin Immunol Immunopathol* **85**:273-81.
443. **Rich, K. C., M. G. Fowler, L. M. Mofenson, R. Abboud, J. Pitt, C. Diaz, I. C. Hanson, E. Cooper, and H. Mendez.** 2000. Maternal and infant factors predicting disease progression in human immunodeficiency virus type 1-infected infants. Women and Infants Transmission Study Group. *Pediatrics* **105**:e8.
444. **Rich, K. C., M. G. Fowler, L. M. Mofenson, R. Abboud, J. Pitt, C. Diaz, I. C. Hanson, E. Cooper, H. Mendez, and W. a. I. T. S. Group.** 2000. Maternal and Infant Factors Predicting Disease Progression in HIV Type 1-Infected Infants. *Pediatrics* **105**:8-20.
445. **Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci U S A* **100**:4144-9.
446. **Riviere, Y., and F. Buseyne.** 1998. Cytotoxic T lymphocytes generation capacity in early life with particular reference to HIV. *Vaccine* **16**:1420-2.
447. **Roederer, M., J. G. Dubs, M. T. Anderson, P. A. Raju, L. A. Herzenberg, and L. A. Herzenberg.** 1995. CD8 naive T cell counts decrease progressively in HIV-infected adults. *J Clin Invest* **95**:2061-6.
448. **Rogel, M. E., L. I. Wu, and M. Emerman.** 1995. The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection. *J Virol* **69**:882-8.
449. **Rollins, N. C., S. M. Filteau, A. Coutsoudis, and A. M. Tomkins.** 2001. Feeding mode, intestinal permeability, and neopterin excretion: a longitudinal study in infants of HIV-infected South African women. *J Acquir Immune Defic Syndr* **28**:132-9.
450. **Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker.** 1997. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278**:1447-50.
451. **Rouas-Freiss, N., R. M. Goncalves, C. Menier, J. Dausset, and E. D. Carosella.** 1997. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytotoxicity. *Proc Natl Acad Sci U S A* **94**:11520-5.

452. Rouet, F., P. Fassinou, A. Inwoley, M. F. Anaky, A. Kouakoussui, C. Rouzioux, S. Blanche, and P. Msellati. 2006. Long-term survival and immuno-virological response of African HIV-1-infected children to highly active antiretroviral therapy regimens. *Aids* 20:2315-9.
453. Rouet, F., C. Sakarovitch, P. Msellati, N. Elenga, C. Montcho, I. Viho, S. Blanche, C. Rouzioux, F. Dabis, and V. Leroy. 2003. Pediatric viral human immunodeficiency virus type 1 RNA levels, timing of infection, and disease progression in African HIV-1-infected children. *Pediatrics* 112:e289.
454. Rousseau, C. M., R. W. Nduati, B. A. Richardson, M. S. Steele, G. C. John-Stewart, D. A. Mbori-Ngacha, J. K. Kreiss, and J. Overbaugh. 2003. Longitudinal analysis of human immunodeficiency virus type 1 RNA in breast milk and of its relationship to infant infection and maternal disease. *J Infect Dis* 187:741-7.
455. Rouzioux, C., D. Costagliola, M. Burgard, S. Blanche, M. J. Mayaux, C. Griscelli, and A. J. Valleron. 1995. Estimated timing of mother-to-child human immunodeficiency virus type 1 (HIV-1) transmission by use of a Markov model. The HIV Infection in Newborns French Collaborative Study Group. *Am J Epidemiol* 142:1330-7.
456. Rowland-Jones, S., J. Sutton, K. Ariyoshi, T. Dong, F. Gotch, S. McAdam, D. Whitby, S. Sabally, A. Gallimore, T. Corrah, and et al. 1995. HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. *Nat Med* 1:59-64.
457. Rowland-Jones, S. L., D. F. Nixon, M. C. Aldhous, F. Gotch, K. Ariyoshi, N. Hallam, J. S. Kroll, K. Froebel, and A. McMichael. 1993. HIV-specific cytotoxic T-cell activity in an HIV-exposed but uninfected infant. *Lancet* 341:860-1.
458. Royce, R. A., A. Sena, W. Cates, Jr., and M. S. Cohen. 1997. Sexual transmission of HIV. *N Engl J Med* 336:1072-8.
459. Ruben, S., A. Perkins, R. Purcell, K. Joung, R. Sia, R. Burghoff, W. A. Haseltine, and C. A. Rosen. 1989. Structural and functional characterization of human immunodeficiency virus tat protein. *J Virol* 63:1-8.
460. Rutherford, G. W., A. R. Lifson, N. A. Hessel, W. W. Darrow, P. M. O'Malley, S. P. Buchbinder, J. L. Barnhart, T. W. Bodecker, L. Cannon, L. S. Doll, and et al. 1990. Course of HIV-I infection in a cohort of homosexual and bisexual men: an 11 year follow up study. *Bmj* 301:1183-8.
461. Ryan, M., L. Gothefors, J. Storsaeter, and K. H. Mills. 1997. Bordetella pertussis-specific Th1/Th2 cells generated following respiratory infection or immunization with an acellular vaccine: comparison of the T cell cytokine profiles in infants and mice. *Dev Biol Stand* 89:297-305.
462. Ryan, M., G. Murphy, L. Gothefors, L. Nilsson, J. Storsaeter, and K. H. Mills. 1997. Bordetella pertussis respiratory infection in children is associated with preferential activation of type 1 T helper cells. *J Infect Dis* 175:1246-50.
463. Sakaguchi, S. 2004. Naturally arising CD4⁺ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22:531-62.
464. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-64.

465. **Salemi, M., K. Strimmer, W. W. Hall, M. Duffy, E. Delaporte, S. Mboup, M. Peeters, and A. M. Vandamme.** 2001. Dating the common ancestor of SIVcpz and HIV-1 group M and the origin of HIV-1 subtypes using a new method to uncover clock-like molecular evolution. *Faseb J* **15**:276-8.
466. **Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cognaux, C. Forceille, G. Muyldermans, C. Verhofstede, G. Burtonboy, M. Georges, T. Imai, S. Rana, Y. Yi, R. J. Smyth, R. G. Collman, R. W. Doms, G. Vassart, and M. Parmentier.** 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**:722-5.
467. **Sandberg, J. K., N. M. Fast, K. A. Jordan, S. N. Furlan, J. D. Barbour, G. Fennelly, J. Dobroszycki, H. M. Spiegel, A. Wiznia, M. G. Rosenberg, and D. F. Nixon.** 2003. HIV-specific CD8+ T cell function in children with vertically acquired HIV-1 infection is critically influenced by age and the state of the CD4+ T cell compartment. *J Immunol* **170**:4403-10.
468. **Sanders, M. E., M. W. Makgoba, S. O. Sharrow, D. Stephany, T. A. Springer, H. A. Young, and S. Shaw.** 1988. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. *J Immunol* **140**:1401-7.
469. **Saper, M. A., P. J. Bjorkman, and D. C. Wiley.** 1991. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *J Mol Biol* **219**:277-319.
470. **Sato, A. I., F. B. Balamuth, K. E. Ugen, W. V. Williams, and D. B. Weiner.** 1994. Identification of CD7 glycoprotein as an accessory molecule in HIV-1-mediated syncytium formation and cellfree infection. *J Immunol* **152**:5142-52.
471. **Saxinger, W. C., P. H. Levine, A. G. Dean, G. de The, G. Lange-Wantzin, J. Moghissi, F. Laurent, M. Hoh, M. G. Sarngadharan, and R. C. Gallo.** 1985. Evidence for exposure to HTLV-III in Uganda before 1973. *Science* **227**:1036-8.
472. **Scarlatti, G., T. Leitner, V. Hodara, E. Halapi, P. Rossi, J. Albert, and E. M. Fenyo.** 1993. Neutralizing antibodies and viral characteristics in mother-to-child transmission of HIV-1. *Aids* **7 Suppl 2**:S45-8.
473. **Schacker, T. W., J. P. Hughes, T. Shea, R. W. Coombs, and L. Corey.** 1998. Biological and virologic characteristics of primary HIV infection. *Ann Intern Med* **128**:613-20.
474. **Schatz, D. G., M. A. Oettinger, and D. Baltimore.** 1989. The V(D)J recombination activating gene, RAG-1. *Cell* **59**:1035-48.
475. **Schim van der Loeff, M. F., P. Aaby, K. Ariyoshi, T. Vincent, A. A. Awasana, C. Da Costa, L. Pembrey, F. Dias, E. Harding, H. A. Weiss, and H. C. Whittle.** 2001. HIV-2 does not protect against HIV-1 infection in a rural community in Guinea-Bissau. *Aids* **15**:2303-10.
476. **Schim van der Loeff, M. F., A. Hansmann, A. A. Awasana, M. O. Ota, D. O'Donovan, R. Sarge-Njie, K. Ariyoshi, P. Milligan, and H. Whittle.** 2003. Survival of HIV-1 and HIV-2 perinatally infected children in The Gambia. *Aids* **17**:2389-94.
477. **Schleiss, M.** 2005. Progress in cytomegalovirus vaccine development. *Herpes* **12**:66-75.

478. **Schleiss, M. R.** 2003. Vertically transmitted herpesvirus infections. *Herpes* 10:4-11.
479. **Schlesinger, Y., D. Reich, A. I. Eidelman, M. S. Schimmel, J. Hassanin, and D. Miron.** 2005. Congenital cytomegalovirus infection in Israel: screening in different subpopulations. *Isr Med Assoc J* 7:237-40.
480. **Schluns, K. S., W. C. Kieper, S. C. Jameson, and L. Lefrancois.** 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* 1:426-32.
481. **Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann.** 1999. Control of viremia in simian immunodeficiency virus infection by CD8⁺ lymphocytes. *Science* 283:857-60.
482. **Schnittman, S. M., S. M. Denning, J. J. Greenhouse, J. S. Justement, M. Baseler, J. Kurtzberg, B. F. Haynes, and A. S. Fauci.** 1990. Evidence for susceptibility of intrathymic T-cell precursors and their progeny carrying T-cell antigen receptor phenotypes TCR alpha beta + and TCR gamma delta + to human immunodeficiency virus infection: a mechanism for CD4⁺ (T4) lymphocyte depletion. *Proc Natl Acad Sci U S A* 87:7727-31.
483. **Schroder, A. R., P. Shinn, H. Chen, C. Berry, J. R. Ecker, and F. Bushman.** 2002. HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* 110:521-9.
484. **Schwartz, O., V. Marechal, S. Le Gall, F. Lemonnier, and J. M. Heard.** 1996. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat Med* 2:338-42.
485. **Schwartz, S., B. K. Felber, D. M. Benko, E. M. Fenyo, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. *J Virol* 64:2519-29.
486. **Scott, G. B., B. E. Buck, J. G. Leterman, F. L. Bloom, and W. P. Parks.** 1984. Acquired immunodeficiency syndrome in infants. *N Engl J Med* 310:76-81.
487. **Scott, Z. A., E. G. Chadwick, L. L. Gibson, M. D. Catalina, M. M. McManus, R. Yogev, P. Palumbo, J. L. Sullivan, P. Britto, H. Gay, and K. Luzuriaga.** 2001. Infrequent detection of HIV-1-specific, but not cytomegalovirus-specific, CD8⁽⁺⁾ T cell responses in young HIV-1-infected infants. *J Immunol* 167:7134-40.
488. **Scott-Algara, D., F. Buseyne, S. Blanche, C. Rouzioux, C. Jouanne, F. Romagne, and Y. Riviere.** 2001. Frequency and phenotyping of human immunodeficiency virus (HIV)-specific CD8⁺ T cells in HIV-infected children, using major histocompatibility complex class I peptide tetramers. *J Infect Dis* 183:1565-73.
489. **Scott-Algara, D., F. Buseyne, F. Porrot, B. Corre, N. Bellal, C. Rouzioux, S. Blanche, and Y. Riviere.** 2005. Not all tetramer binding CD8⁺ T cells can produce cytokines and chemokines involved in the effector functions of virus-specific CD8⁺ T lymphocytes in HIV-1 infected children. *J Clin Immunol* 25:57-67.
490. **Semba, R. D., N. Kumwenda, D. R. Hoover, T. E. Taha, T. C. Quinn, L. Mtimavalye, R. J. Biggar, R. Broadhead, P. G. Miotti, L. J. Sokoll, L. van der Hoeven, and J. D. Chipangwi.** 1999. Human immunodeficiency virus

- load in breast milk, mastitis, and mother-to-child transmission of human immunodeficiency virus type 1. *J Infect Dis* **180**:93-8.
491. **Sercarz, E. E., and E. Maverakis.** 2003. Mhc-guided processing: binding of large antigen fragments. *Nat Rev Immunol* **3**:621-9.
 492. **Shaffer, N., R. Chuachoowong, P. A. Mock, C. Bhadrakom, W. Siriwasin, N. L. Young, T. Chotpitayasunondh, S. Chearskul, A. Roongpisuthipoong, P. Chinayon, J. Karon, T. D. Mastro, and R. J. Simonds.** 1999. Short-course zidovudine for perinatal HIV-1 transmission in Bangkok, Thailand: a randomised controlled trial. *Lancet* **353**:773-780.
 493. **Shankar, P., M. Russo, B. Harnisch, M. Patterson, P. Skolnik, and J. Lieberman.** 2000. Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. *Blood* **96**:3094-101.
 494. **Shannon, K., E. Ball, R. L. Wasserman, F. K. Murphy, J. Luby, and G. R. Buchanan.** 1983. Transfusion-associated cytomegalovirus infection and acquired immune deficiency syndrome in an infant. *J Pediatr* **103**:859-63.
 495. **Shapiro, R. L., S. Lockman, S. Kim, L. Smeaton, J. T. Rahkola, I. Thior, C. Wester, C. Moffat, P. Arimi, P. Ndase, A. Asmelash, L. Stevens, M. Montano, J. Makhema, M. Essex, and E. N. Janoff.** 2007. Infant morbidity, mortality, and breast milk immunologic profiles among breast-feeding HIV-infected and HIV-uninfected women in Botswana. *J Infect Dis* **196**:562-9.
 496. **Shearer, W. T., T. C. Quinn, P. LaRussa, J. F. Lew, L. Mofenson, S. Almy, K. Rich, E. Handelsman, C. Diaz, M. Pagano, V. Smeriglio, and L. A. Kalish.** 1997. Viral load and disease progression in infants infected with human immunodeficiency virus type 1. Women and Infants Transmission Study Group. *N Engl J Med* **336**:1337-42.
 497. **Shearer, W. T., H. M. Rosenblatt, R. S. Gelman, R. Oyomopito, S. Plaeger, E. R. Stiehm, D. W. Wara, S. D. Douglas, K. Luzuriaga, E. J. McFarland, R. Yogeve, M. H. Rathore, W. Levy, B. L. Graham, and S. A. Spector.** 2003. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. *J Allergy Clin Immunol* **112**:973-80.
 498. **Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**:646-50.
 499. **Sherman, G. G., L. E. Scott, J. S. Galpin, L. Kuhn, C. T. Tiemessen, K. Simmank, S. Meddows-Taylor, and T. M. Meyers.** 2002. CD38 expression on CD8(+) T cells as a prognostic marker in vertically HIV-infected pediatric patients. *Pediatr Res* **51**:740-5.
 500. **Sherry, B., G. Zybarth, M. Alfano, L. Dubrovsky, R. Mitchell, D. Rich, P. Ulrich, R. Bucala, A. Cerami, and M. Bukrinsky.** 1998. Role of cyclophilin A in the uptake of HIV-1 by macrophages and T lymphocytes. *Proc Natl Acad Sci U S A* **95**:1758-63.
 501. **Sieg, S. F., C. V. Harding, and M. M. Lederman.** 2001. HIV-1 infection impairs cell cycle progression of CD4(+) T cells without affecting early activation responses. *J Clin Invest* **108**:757-64.
 502. **Simonon, A., G. A. Mulder-Kampinga, P. van der Perre, E. Karita, P. Msellati, C. Kuiken, and J. Goudsmit.** 1997. Evolution of HIV subtype A in women seroconverting post partum and in their offspring post-natally infected by ingestion of breast milk. *J Gen Virol* **78**:2235-2233.

503. **Simonsen, J. N., D. W. Cameron, M. N. Gakinya, J. O. Ndinya-Achola, L. J. D'Costa, P. Karasira, M. Cheang, A. R. Ronald, P. Piot, and F. A. Plummer.** 1988. Human immunodeficiency virus infection among men with sexually transmitted diseases. Experience from a center in Africa. *N Engl J Med* **319**:274-8.
504. **Skowronski, J., D. Parks, and R. Mariani.** 1993. Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. *Embo J* **12**:703-13.
505. **Slifka, M., J. Blattman, D. Sourdive, F. Liu, D. Huffman, T. Wolfe, A. Hughes, M. Oldstone, R. Ahmed, and M. G. von Herrath.** 2003. Preferential escape of subdominant CD8+ T cells during negative selection results in an altered antiviral T cell hierarchy. *J Immunol* **170**:1231-1239.
506. **Slyker, J. A., B. L. Lohman, D. A. Mbori-Ngacha, M. Reilly, E. G. Wee, T. Dong, A. J. McMichael, S. L. Rowland-Jones, T. Hanke, and G. John-Stewart.** 2005. Modified vaccinia Ankara expressing HIVA antigen stimulates HIV-1-specific CD8 T cells in ELISpot assays of HIV-1 exposed infants. *Vaccine* **23**:4711-9.
507. **Smithies, O.** 1963. Gamma-Globulin Variability: a Genetic Hypothesis. *Nature* **199**:1231-6.
508. **Snow, R. W., J. R. Armstrong, D. Forster, M. T. Winstanley, V. M. Marsh, C. R. Newton, C. Waruiru, I. Mwangi, P. A. Winstanley, and K. Marsh.** 1992. Childhood deaths in Africa: uses and limitations of verbal autopsies. *Lancet* **340**:351-5.
509. **Soilleux, E. J., L. S. Morris, B. Lee, S. Pohlmann, J. Trowsdale, R. W. Doms, and N. Coleman.** 2001. Placental expression of DC-SIGN may mediate intrauterine vertical transmission of HIV. *J Pathol* **195**:586-92.
510. **Spector, S. A., K. Hsia, M. Crager, M. Pilcher, S. Cabral, and M. J. Stempien.** 1999. Cytomegalovirus (CMV) DNA load is an independent predictor of CMV disease and survival in advanced AIDS. *J Virol* **73**:7027-30.
511. **Spector, S. A., G. F. McKinley, J. P. Lalezari, T. Samo, R. Andruczk, S. Follansbee, P. D. Sparti, D. V. Havlir, G. Simpson, W. Buhles, R. Wong, and M. Stempien.** 1996. Oral ganciclovir for the prevention of cytomegalovirus disease in persons with AIDS. Roche Cooperative Oral Ganciclovir Study Group. *N Engl J Med* **334**:1491-7.
512. **Spector, S. A., R. Wong, K. Hsia, M. Pilcher, and M. J. Stempien.** 1998. Plasma cytomegalovirus (CMV) DNA load predicts CMV disease and survival in AIDS patients. *J Clin Invest* **101**:497-502.
513. **Sperling, R. S., D. E. Shapiro, R. W. Coombs, J. A. Todd, S. A. Herman, G. D. McSherry, M. J. O'Sullivan, R. B. Van Dyke, E. Jimenez, C. Rouzioux, P. M. Flynn, and J. L. Sullivan.** 1996. Maternal viral load, zidovudine treatment, and the risk of transmission of human immunodeficiency virus type 1 from mother to infant. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. *N Engl J Med* **335**:1621-9.
514. **Spiegel, H. M., G. S. Ogg, E. DeFalcon, M. E. Sheehy, S. Monard, P. A. Haslett, G. Gillespie, S. M. Donahoe, H. Pollack, W. Borkowsky, A. J. McMichael, and D. F. Nixon.** 2000. Human immunodeficiency virus type 1- and cytomegalovirus-specific cytotoxic T lymphocytes can persist at high frequency for prolonged periods in the absence of circulating peripheral CD4(+) T cells. *J Virol* **74**:1018-22.

515. **Sprecher, S., G. Soumenkoff, F. Puissant, and M. Degueldre.** 1986. Vertical transmission of HIV in 15-week fetus. *Lancet* **2**:288-9.
516. **St Louis, M. E., M. Kamenga, C. Brown, A. M. Nelson, T. Manzila, V. Batter, F. Behets, U. Kabagabo, R. W. Ryder, M. Oxtoby, and et al.** 1993. Risk for perinatal HIV-1 transmission according to maternal immunologic, virologic, and placental factors. *Jama* **269**:2853-9.
517. **Stagno, S., and G. A. Cloud.** 1994. Working parents: the impact of day care and breast-feeding on cytomegalovirus infections in offspring. *Proc Natl Acad Sci U S A* **91**:2384-9.
518. **Stagno, S., R. F. Pass, G. Cloud, W. J. Britt, R. E. Henderson, P. D. Walton, D. A. Veren, F. Page, and C. A. Alford.** 1986. Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. *Jama* **256**:1904-8.
519. **Stagno, S., R. F. Pass, M. E. Dworsky, R. E. Henderson, E. G. Moore, P. D. Walton, and C. A. Alford.** 1982. Congenital cytomegalovirus infection: The relative importance of primary and recurrent maternal infection. *N Engl J Med* **306**:945-9.
520. **Stanley, S. K., S. W. Kessler, J. S. Justement, S. M. Schnittman, J. J. Greenhouse, C. C. Brown, L. Musongela, K. Musey, B. Kapita, and A. S. Fauci.** 1992. CD34+ bone marrow cells are infected with HIV in a subset of seropositive individuals. *J Immunol* **149**:689-97.
521. **Sterling, T. R., D. Vlahov, J. Astemborski, D. R. Hoover, J. B. Margolick, and T. C. Quinn.** 2001. Initial plasma HIV-1 RNA levels and progression to AIDS in women and men. *N Engl J Med* **344**:720-5.
522. **Stern, L. J., J. H. Brown, T. S. Jardetzky, J. C. Gorga, R. G. Urban, J. L. Strominger, and D. C. Wiley.** 1994. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* **368**:215-21.
523. **Su, B., G. Sun, D. Lu, J. Xiao, F. Hu, R. Chakraborty, R. Deka, and L. Jin.** 2000. Distribution of three HIV-1 resistance-conferring polymorphisms (SDF1-3'A, CCR2-641, and CCR5-delta32) in global populations. *Eur J Hum Genet* **8**:975-9.
524. **Sutton, J., S. Rowland-Jones, W. Rosenberg, D. Nixon, F. Gotch, X. M. Gao, N. Murray, A. Spoonas, P. Driscoll, M. Smith, and et al.** 1993. A sequence pattern for peptides presented to cytotoxic T lymphocytes by HLA B8 revealed by analysis of epitopes and eluted peptides. *Eur J Immunol* **23**:447-53.
525. **Svarovskaia, E. S., H. Xu, J. L. Mbisa, R. Barr, R. J. Gorelick, A. Ono, E. O. Freed, W. S. Hu, and V. K. Pathak.** 2004. Human apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) is incorporated into HIV-1 virions through interactions with viral and nonviral RNAs. *J Biol Chem* **279**:35822-8.
526. **Sylwester, A. W., B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti, P. R. Sleath, K. H. Grabstein, N. A. Hosken, F. Kern, J. A. Nelson, and L. J. Picker.** 2005. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med* **202**:673-85.
527. **Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher.** 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**:655-69.

528. **Taha, T. E., and R. H. Gray.** 2000. Genital tract infections and perinatal transmission of HIV. *Ann N Y Acad Sci* **918**:84-98.
529. **Takeda, K., and S. Akira.** 2005. Toll-like receptors in innate immunity. *Int Immunol* **17**:1-14.
530. **Tao, X., S. Constant, P. Jorritsma, and K. Bottomly.** 1997. Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *J Immunol* **159**:5956-63.
531. **Tao, X., C. Grant, S. Constant, and K. Bottomly.** 1997. Induction of IL-4-producing CD4+ T cells by antigenic peptides altered for TCR binding. *J Immunol* **158**:4237-44.
532. **Thali, M., A. Bukovsky, E. Kondo, B. Rosenwirth, C. T. Walsh, J. Sodroski, and H. G. Gottlinger.** 1994. Functional association of cyclophilin A with HIV-1 virions. *Nature* **372**:363-5.
533. **Thananchai, H., G. Gillespie, M. P. Martin, A. Bashirova, N. Yawata, M. Yawata, P. Easterbrook, D. W. McVicar, K. Maenaka, P. Parham, M. Carrington, T. Dong, and S. Rowland-Jones.** 2007. Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. *J Immunol* **178**:33-7.
534. **Thobakgale, C. F., D. Ramduth, S. Reddy, N. Mkhwanazi, C. de Pierres, E. Moodley, W. Mphatswe, N. Blanckenberg, A. Cengimbo, A. Prendergast, G. Tudor-Williams, K. Dong, P. Jeena, G. Kindra, R. Bobat, H. Coovadia, P. Kiepiela, B. D. Walker, and P. J. Goulder.** 2007. Hiv-Specific Cd8+ T Cell Activity Is Detectable from Birth in the Majority of in Utero Infected Infants. *J Virol*.
535. **Tonegawa, S.** 1976. Reiteration frequency of immunoglobulin light chain genes: further evidence for somatic generation of antibody diversity. *Proc Natl Acad Sci U S A* **73**:203-7.
536. **Tormey, D. C., R. C. Imrie, and G. C. Mueller.** 1972. Identification of transferrin as a lymphocyte growth promoter in human serum. *Exp. Cell Res.* **74**:163-169.
537. **Townsend, A., C. Ohlen, J. Bastin, H. G. Ljunggren, L. Foster, and K. Karre.** 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature* **340**:443-8.
538. **Townsend, A., and J. Trowsdale.** 1993. The transporters associated with antigen presentation. *Semin Cell Biol* **4**:53-61.
539. **Townsend, A. R., F. M. Gotch, and J. Davey.** 1985. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell* **42**:457-67.
540. **Townsend, A. R., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael.** 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44**:959-68.
541. **Townsend, A. R., and J. J. Skehel.** 1984. The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells. *J Exp Med* **160**:552-63.
542. **Trautmann, L., L. Janbazian, N. Chomont, E. A. Said, S. Gimmig, B. Bessette, M. R. Boulassel, E. Delwart, H. Sepulveda, R. S. Balderas, J. P. Routy, E. K. Haddad, and R. P. Sekaly.** 2006. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* **12**:1198-202.

543. **Trowbridge, I. S., and M. B. Omary.** 1981. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc Natl Acad Sci U S A* **78**:3039-43.
544. **Tsegaye, A., D. Wolday, S. Otto, B. Petros, T. Assefa, T. Alebachew, E. Hailu, F. Adugna, W. Measho, W. Dorigo, A. L. Fontanet, D. van Baarle, and F. Miedema.** 2003. Immunophenotyping of blood lymphocytes at birth, during childhood, and during adulthood in HIV-1-uninfected Ethiopians. *Clin Immunol* **109**:338-46.
545. **Tsunemi, S., T. Iwasaki, T. Imado, S. Higasa, E. Kakishita, T. Shirasaka, and H. Sano.** 2005. Relationship of CD4+CD25+ regulatory T cells to immune status in HIV-infected patients. *Aids* **19**:879-86.
546. **Tu, W., S. Chen, M. Sharp, C. Dekker, A. M. Manganello, E. C. Tongson, H. T. Maecker, T. H. Holmes, Z. Wang, G. Kemble, S. Adler, A. Arvin, and D. B. Lewis.** 2004. Persistent and selective deficiency of CD4+ T cell immunity to cytomegalovirus in immunocompetent young children. *J Immunol* **172**:3260-7.
547. **UNAIDS.** 2006. AIDS epidemic update. UNAIDS.
548. **UNAIDS.** 2002. Kenya HIV/AIDS Country Report. UNAIDS.
549. **UNAIDS, and WHO** Sep 19, 2007 2006, posting date. Report on the global AIDS epidemic: Executive summary. [Online.]
550. **Urso, P., and T. Makinodan.** 1963. The Roles of Cellular Division and Maturation in the Formation of Precipitating Antibody. *J Immunol* **90**:897-907.
551. **Van de Perre, P., D. Munyambuga, G. Zissis, J. P. Butlzer, D. Nzaramba, and N. Clumeck.** 1985. Antibody to HTLV-III in blood donors in central Africa. *Lancet* **1**:336-7.
552. **Van de Perre, P., A. Simonon, D. G. Hitimana, F. Dabis, P. Msellati, B. Mukamabano, J. B. Butera, C. Van Goethem, E. Karita, and P. Lepage.** 1993. Infective and anti-infective properties of breastmilk from HIV-1-infected women. *Lancet* **341**:914-8.
553. **van der Sande, M. A., S. Kaye, D. J. Miles, P. Waight, D. J. Jeffries, O. O. Ojuola, M. Palmero, M. Pinder, J. Ismaili, K. L. Flanagan, A. A. Aveika, A. Zaman, S. Rowland-Jones, S. J. McConkey, H. C. Whittle, and A. Marchant.** 2007. Risk factors for and clinical outcome of congenital cytomegalovirus infection in a peri-urban West-African birth cohort. *PLoS ONE* **2**:e492.
554. **Van Rompay, K. K., C. J. Berardi, S. Dillard-Telm, R. P. Tarara, D. R. Canfield, C. R. Valverde, D. C. Montefiori, K. S. Cole, R. C. Montelaro, C. J. Miller, and M. L. Marthas.** 1998. Passive immunization of newborn rhesus macaques prevents oral simian immunodeficiency virus infection. *J Infect Dis* **177**:1247-59.
555. **Vidal, N., M. Peeters, C. Mulanga-Kabeya, N. Nzilambi, D. Robertson, W. Ilunga, H. Sema, K. Tshimanga, B. Bongo, and E. Delaporte.** 2000. Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. *J Virol* **74**:10498-507.
556. **Vilches, C., and P. Parham.** 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* **20**:217-51.

557. **Vinuesa, C. G., S. G. Tangye, B. Moser, and C. R. Mackay.** 2005. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* **5**:853-65.
558. **Violari, A., M. Cotton, D. Gibb, A. Babiker, J. Steyn, P. Jean-Phillip, J. McIntyre, and C. S. Team.** 2007. Presented at the 4th IAS Conference on HIV Pathogenesis Treatment and Prevention, Sydney, Australia.
559. **Vochem, M., K. Hamprecht, G. Jahn, and C. P. Speer.** 1998. Transmission of cytomegalovirus to preterm infants through breast milk. *Pediatr Infect Dis J* **17**:53-8.
560. **Walker, B. D., S. Chakrabarti, B. Moss, T. J. Paradis, T. Flynn, A. G. Durno, R. S. Blumberg, J. C. Kaplan, M. S. Hirsch, and R. T. Schooley.** 1987. HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* **328**:345-8.
561. **Wamalwa, D. C., C. Farquhar, E. M. Obimbo, S. Selig, D. A. Mbori-Ngacha, B. A. Richardson, J. Overbaugh, S. Emery, G. Wariua, C. Gichuhi, R. Bosire, and G. John-Stewart.** 2007. Early response to highly active antiretroviral therapy in HIV-1-infected Kenyan children. *J Acquir Immune Defic Syndr* **45**:311-7.
562. **Wang, X., D. Y. Huang, S. M. Huong, and E. S. Huang.** 2005. Integrin alphavbeta3 is a coreceptor for human cytomegalovirus. *Nat Med* **11**:515-21.
563. **Wang, X., S. M. Huong, M. L. Chiu, N. Raab-Traub, and E. S. Huang.** 2003. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. *Nature* **424**:456-61.
564. **Wasik, T. J., P. P. Jagodzinski, E. M. Hyjek, J. Wustner, G. Trinchieri, H. W. Lischner, and D. Kozbor.** 1997. Diminished HIV-specific CTL activity is associated with lower type 1 and enhanced type 2 responses to HIV-specific peptides during perinatal HIV infection. *J Immunol* **158**:6029-36.
565. **Wasik, T. J., A. Wierzbicki, V. E. Whiteman, G. Trinchieri, H. W. Lischner, and D. Kozbor.** 2000. Association between HIV-specific T helper responses and CTL activities in pediatric AIDS. *Eur J Immunol* **30**:117-27.
566. **Wasik, T. J., A. Wierzbicki, V. E. Whiteman, G. Trinchieri, H. W. Lischner, and D. Kozbor.** 2000. Association between HIV-specific T helper responses and CTL activities in pediatric AIDS. *Eur J Immunol* **30**:117-27.
567. **Weber, D. A., B. D. Evavold, and P. E. Jensen.** 1996. Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* **274**:618-20.
568. **Wegmann, T. G., H. Lin, L. Guilbert, and T. R. Mosmann.** 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* **14**:353-6.
569. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. *Nature* **422**:307-12.
570. **Weinberg, A., C. Tierney, M. A. Kendall, R. J. Bosch, J. Patterson-Bartlett, A. Erice, M. S. Hirsch, and B. Polsky.** 2006. Cytomegalovirus-specific immunity and protection against viremia and disease in HIV-infected patients in the era of highly active antiretroviral therapy. *J Infect Dis* **193**:488-93.

571. **Weinberg, A., A. A. Wiznia, B. J. Lafleur, S. Shah, and M. J. Levin.** 2006. Cytomegalovirus-specific cell-mediated immunity in HIV-infected children on HAART. *AIDS Res Hum Retroviruses* **22**:283-8.
572. **Weinberger, B., L. Lazuardi, I. Weiskirchner, M. Keller, C. Neuner, K. H. Fischer, B. Neuman, R. Wurzner, and B. Grubeck-Loebenstien.** 2007. Healthy aging and latent infection with CMV lead to distinct changes in CD8+ and CD4+ T-cell subsets in the elderly. *Hum Immunol* **68**:86-90.
573. **Westendorp, M. O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K. M. Debatin, and P. H. Krammer.** 1995. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature* **375**:497-500.
574. **White, G. P., P. M. Watt, B. J. Holt, and P. G. Holt.** 2002. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells. *J Immunol* **168**:2820-7.
575. **WHO.** 2006.
576. **WHO** 2006, posting date. Antiretroviral therapy of HIV infection in infants and children: Towards universal access. [Online.]
577. **WHO** 2006, posting date. WHO HIV and infant feeding technical consultation held on behalf of the inter-agency task team on prevention of HIV infections in pregnant women, mothers and their infants. [Online.]
578. **Wikby, A., B. Johansson, J. Olsson, S. Lofgren, B. O. Nilsson, and F. Ferguson.** 2002. Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. *Exp Gerontol* **37**:445-53.
579. **Willey, R. L., F. Maldarelli, M. A. Martin, and K. Strebel.** 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. *J Virol* **66**:7193-200.
580. **Willumsen, J. F., S. M. Filteau, A. Coutoudis, K. E. Uebel, M. L. Newell, and A. M. Tomkins.** 2000. Subclinical mastitis as a risk factor for mother-infant HIV transmission. *Adv Exp Med Biol* **478**:211-23.
581. **Wilson, A., H. R. MacDonald, and F. Radtke.** 2001. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med* **194**:1003-12.
582. **Wilson, C. C., S. A. Kalams, B. M. Wilkes, D. J. Ruhl, F. Gao, B. H. Hahn, I. C. Hanson, K. Luzuriaga, S. Wolinsky, R. Koup, S. P. Buchbinder, R. P. Johnson, and B. D. Walker.** 1997. Overlapping epitopes in human immunodeficiency virus type 1 gp120 presented by HLA A, B, and C molecules: effects of viral variation on cytotoxic T-lymphocyte recognition. *J Virol* **71**:1256-64.
583. **Wilson, J. D., G. S. Ogg, R. L. Allen, C. Davis, S. Shaunak, J. Downie, W. Dyer, C. Workman, S. Sullivan, A. J. McMichael, and S. L. Rowland-Jones.** 2000. Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. *Aids* **14**:225-33.
584. **Wohl, D. A., D. Zeng, P. Stewart, N. Glomb, T. Alcorn, S. Jones, J. Handy, S. Fiscus, A. Weinberg, D. Gowda, and C. van der Horst.** 2005. Cytomegalovirus viremia, mortality, and end-organ disease among patients with AIDS receiving potent antiretroviral therapies. *J Acquir Immune Defic Syndr* **38**:538-44.
585. **Wolthers, K. C., G. Bea, A. Wisman, S. A. Otto, A. M. de Roda Husman, N. Schaft, F. de Wolf, J. Goudsmit, R. A. Coutinho, A. G. van der Zee, L.**

- Meyaard, and F. Miedema. 1996. T cell telomere length in HIV-1 infection: no evidence for increased CD4+ T cell turnover. *Science* **274**:1543-7.
586. Wong, J. K., M. Hezareh, H. F. Gunthard, D. V. Havlir, C. C. Ignacio, C. A. Spina, and D. D. Richman. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* **278**:1291-5.
587. Worku, S., B. Christensson, A. Bjorkman, and D. Islam. 1997. Higher proportion of CD8+ T cells in the blood in healthy adults from Ethiopia and Bangladesh compared with Sweden. *Trans R Soc Trop Med Hyg* **91**:618-22.
588. Wu, X., A. B. Parast, B. A. Richardson, R. Nduati, G. John-Stewart, D. Mbori-Ngacha, S. M. Rainwater, and J. Overbaugh. 2006. Neutralization escape variants of human immunodeficiency virus type 1 are transmitted from mother to infant. *J Virol* **80**:835-44.
589. Wyatt, R., and J. Sodroski. 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**:1884-8.
590. Xu, X. N., G. R. Screaton, F. M. Gotch, T. Dong, R. Tan, N. Almond, B. Walker, R. Stebbings, K. Kent, S. Nagata, J. E. Stott, and A. J. McMichael. 1997. Evasion of cytotoxic T lymphocyte (CTL) responses by nef-dependent induction of Fas ligand (CD95L) expression on simian immunodeficiency virus-infected cells. *J Exp Med* **186**:7-16.
591. Yagi, M. J., F. N. Chu, J. D. Jiang, J. Wallace, P. Mason, Y. Liu, J. Carafa, and J. G. Bekesi. 1992. Increases in soluble CD8 antigen in plasma, and CD8+ and CD8+CD38+ cells in human immunodeficiency virus type-1 infection. *Clin Immunol Immunopathol* **63**:126-34.
592. Yoshino, T., E. Kondo, L. Cao, K. Takahashi, K. Hayashi, S. Nomura, and T. Akagi. 1994. Inverse expression of bcl-2 protein and Fas antigen in lymphoblasts in peripheral lymph nodes and activated peripheral blood T and B lymphocytes. *Blood* **83**:1856-61.
593. Young, H. A., and K. J. Hardy. 1995. Role of interferon-gamma in immune cell regulation. *J Leukoc Biol* **58**:373-81.
594. Young, J. D., H. Hengartner, E. R. Podack, and Z. A. Cohn. 1986. Purification and characterization of a cytolytic pore-forming protein from granules of cloned lymphocytes with natural killer activity. *Cell* **44**:849-59.
595. Yu, X., Y. Yu, B. Liu, K. Luo, W. Kong, P. Mao, and X. F. Yu. 2003. Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* **302**:1056-60.
596. Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* **188**:2205-13.
597. Zauli, G., D. Gibellini, P. Secchiero, H. Dutartre, D. Olive, S. Capitani, and Y. Collette. 1999. Human immunodeficiency virus type 1 Nef protein sensitizes CD4(+) T lymphoid cells to apoptosis via functional upregulation of the CD95/CD95 ligand pathway. *Blood* **93**:1000-10.
598. Zhang, H., B. Yang, R. J. Pomerantz, C. Zhang, S. C. Arunachalam, and L. Gao. 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* **424**:94-8.
599. Zhu, T., L. Corey, Y. Hwangbo, J. M. Lee, G. H. Learn, J. I. Mullins, and M. J. McElrath. 2003. Persistence of extraordinarily low levels of genetically homogeneous human immunodeficiency virus type 1 in exposed seronegative individuals. *J Virol* **77**:6108-16.

- 600. **Zhu, T., B. T. Korber, A. J. Nahmias, E. Hooper, P. M. Sharp, and D. D. Ho.** 1998. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. *Nature* **391**:594-7.
- 601. **Zinkernagel, R. M., and P. C. Doherty.** 1974. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature* **251**:547-8.
- 602. **Zinkernagel, R. M., and P. C. Doherty.** 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**:701-2.